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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **238th Meeting** *of the Society took the form of a Joint Discussion with the Pathological Society of Great Britain and Ireland on 'Cancer', and was held at the Royal Society of Medicine, London, W.* 1, *on Saturday,* 16 *December* 1944. *The following communications were given:*

OESTROGENS AND MALIGNANT DISEASE (MORNING SESSION)

Synthetic Oestrogens in Cancer. By E. C. DODDS

The name 'Oestrogen', as Parkes has pointed out, limits our conception of the biological activity of these compounds. We tend to think of them as only producing oestrus in the small laboratory rodent, whereas, in addition to activity on the whole of the female genital tract, they also inhibit the anterior lobe of the pituitary. Parkes, therefore, suggested the name 'gynaecogen' in place of oestrogen. These compounds can be classified under three headings:

(1) Natural oestrogens: oestrone, oestradiol, oestriol, and their simple esters.

(2) Modified natural oestrogens: ethinyl oestradiol, where an ethinyl group has been introduced at the C_{17} atom.

(3) Synthetic oestrogens. These can be considered under two main headings: (*a*) derivatives of normal hexane, and (*b*) derivatives of ethylene.

(*a*) Hexoestrol, stilboestrol, dienoestrol.

(*b*) Triphenyl ethylene and its derivatives.

The group (*a*) possess greater activity than the naturally occurring hormones, whilst group (*b*) are many times weaker. Group (*a*) are all highly active by mouth, in contradistinction to the naturally occurring substances.

It is not proposed here to attempt to review completely the work done with this group of compounds in relation to cancer. It is perhaps a fair statement that they have no clear-cut or striking influence on the rate of growth, take, or the fate of transplanted animal tumours. Their carcinogenic activity differs from that of the condensed carbon-ring carcinogens, in that they will not produce cancer locally when painted on the skin of mice. They will, however, unlike the classical carcinogens, cause, when injected into suitable mice, an increase in the incident of mammary carcinoma.

The first evidence that these compounds could play a part in the treatment of cancer arose from observations on carcinoma of the prostate. Subsequent speakers will develop the various arguments leading to the trial of this treatment, and others will testify as to its success.

The greater majority of cases show complete relief of symptoms, and can be maintained symptom-free for long periods. The word 'cure' must not, of course, be used, but the relief of suffering is of the greatest importance. This is the first time that any form of cancer has been favourably affected by a medicine given by mouth.

The mode of action is still obscure, but three possibilities present themselves:

(1) Inhibition of testosterone secretion by the testis.

(2) Inhibition of secretion of gonadotrophin by the anterior lobe of the pituitary.

(3) Direct action on the growth.

It is not yet possible to assign the relative importance to these three possible factors, but the fact that compounds of such widely differing chemical structure as oestradiol and stilboestrol are both capable of causing symptomatic relief, points to the activity lying in their oestrogenic activity rather than their chemical structure.

Wide empirical use has been made of this type of treatment in every type of cancer, but the reported results are of doubtful value owing to their haphazard nature. There does, however, appear to emerge the idea that favourable results may be obtained in a very limited percentage of cases of carcinoma of the breast. Even dramatic results have been published. A fully controlled investigation is being undertaken under the auspices of the Royal Society of Medicine.

In any case the synthetic oestrogens have provided the first clear-cut beneficial effect of any synthetic drug on any form of cancer, and this has provided a new approach to the whole problem. The importance lies in both the practical and theoretical fields.

Inhibition of Growth by Amino-*s*-Diarylethylenes. By A. HADDOW, R. J. C. HARRIS and G. A. R. KON

In attempts to define the molecular conditions for growth-inhibitory action, various triarylethylenes and analogues were examined. Activity was detected in 9-(*p*-dimethylaminobenzal)-fluorene, following which it was decided to test the simpler types represented by basically substituted stilbenes. 4-Dimethylaminostilbene (I; 50 mg./kg.) retarded body growth and tumour growth (Walker carcinoma) in the rat, although this result could be shown only within narrow limits, higher doses producing marked toxic effects. All these effects were manifested to a considerably less extent in mice (Crocker sarcoma, C_{63} carcinoma and spontaneous tumours), and the results indicate a species difference reminiscent of the action of 2-acetylaminofluorene, which is growth-inhibitory and carcinogenic in the rat, but much less active in the mouse.

Inhibitory activity in the rat is retained when the dialkylamino group is transferred from the *para-* to the *ortho-* position, but is absent in *meta*-dimethylaminostilbene. Activity is annulled by *pp'*-disubstitution, by hydrogenation or extension of the ethylene chain, and by alkyl substitution of the ethylene hydrogen atoms. Dissociation of toxicity from inhibitory activity is shown in 4-dimethylamino-2'-methylstilbene, and in α-(4-dimethylaminophenyl)-β-(α-naphthyl)-ethylene, which may produce a fifty-fold reduction in rate of growth of the Walker tumour, without general toxic effects. α-(4-Dimethylaminophenyl)-β-(β-naphthyl)-ethylene is practically inactive.

It is an interesting coincidence that these compounds are derivatives of stilbene. However, the new compounds are not oestrogenic, and the molecular requirements for their inhibitory effects are clearly distinct from those which govern oestrogenicity. It is therefore probable that the interference with growth which they produce arises in a manner other than that responsible for the inhibitory effects of the synthetic oestrogens. It was already known that the inhibition due to oestrogens probably differs from the retardation brought about by non-oestrogenic carcinogens. Thus the former may be neutralized by growth hormone:* such neutralization does not apparently occur in the case of the carcinogenic hydrocarbons.

There is no evidence that the effects can be ascribed to deprivation of essential sulphur-containing amino-acids by mercapturic acid formation, since administration of 4-dimethylaminostilbene, sufficient to produce cessation of growth, does not lead to any increased excretion of neutral sulphur. It appears likely that the action upon growth is the result of a more direct, and perhaps competitive, disturbance in cell metabolism. Regarding the essential carbon skeleton, it is possibly significant that certain of the most potent carcinogenic and inhibitory compounds hitherto reported embody the configuration of a *trans*-αβ-diarylethylene, and that this feature may be absent in their inactive structural isomers.

Histological and cytological alterations are produced in the Walker carcinoma by the most active of the compounds now described, but it is improbable that they are specific,

since similar changes have been observed in human tumours under treatment with stilboestrol.

Representative compounds in the present series are under test for carcinogenic activity.

* Griffiths, M. & Young, F. G. (1942). *J. Endocrinol.* 3, 96.

The Evolution of Mouse Mammary Cancer; the Roles of Milk Factor and Oestrogen. By GEORGIANA M. BONSER

Microscopical study of the evolution of mouse mammary cancer (on the lines of what has already been done in the human by Muir, Cheatle and others) has led to some clarification of the respective roles of the milk factor and oestrogen.

Mouse mammary cancer arises by three processes:

(1) Intra-acinous carcinoma, resulting from a malignant change in the actual lining cells of the acini; the first, most frequent and most important site. Intra-acinous carcinoma occurs in proliferated but not necessarily cystic acini; the degree of proliferation required is extremely variable and may be very slight.

(2) Malignant acinar proliferation, consisting in the formation of groups of closely packed acini, the epithelium of which is in a state of great activity; not very common and always associated with (1).

(3) Intraduct carcinoma, which is uncommon but does occur associated with (1).

By addition of milk factor to resistant males and subsequent oestrogen treatment, the following comparisons with normal mice were made:

(1) Acinar proliferation was of greater degree and intensity and malignant acinar proliferation only occurred where milk factor was present. The difference was more marked in one strain than in the other tested.

(2) Intra-acinous carcinoma was seen in all but one of the high proportion of mice which had received milk factor and subsequently developed mammary cancer.

(3) Fully developed mammary cancer occurred only in mice with milk factor.

Conversely, by withdrawal of milk factor from susceptible males and subsequent oestrogen treatment, it was observed that:

(1) Acinar proliferation was of greater degree and intensity in normal mice, but the difference was less marked than in the converse condition.

(2) Malignant acinar proliferation occurred only in normal mice and in the two foster-nursed mice which subsequently developed mammary cancer.

(3) Intra-acinous carcinoma occurred in nearly all of the high proportion of normal mice which had fully developed cancers but in only two foster-nursed mice, one of which had mammary cancer.

The conclusions to be drawn from these observations are that the milk factor acts mainly on the acini, permitting a higher degree of acinar proliferation than oestrogen alone and leading to intra-acinous carcinoma and fully developed cancer. The association between the two latter conditions is a very close one; the relation between the degree of acinar proliferation and intra-acinous carcinoma is not so clear.

From previous studies it is known that oestrogen causes growth of breast tissue from a simple vestigial tube to a complicated system of ducts and hyperplastic acini. Unless the lining cells are prepared or sensitized by the previous addition of milk factor, the process does not proceed to malignancy. The conception of sensitizing and developing factors in carcinogenesis has been worked out in relation to skin cancer by Mottram and to thyroid cancer by Bielschowsky. In the case of the breast it might be postulated

that the milk factor supplies both the carcinogenic agent and the sensitizing factor, the oestrogen being the developing factor. This idea is supported by some experiments by Orr in which the milk factor was replaced by methylcholanthrene.

Intraduct carcinoma is the common first cancerous change in the human breast, the ducts affected being terminal. Intra-acinous carcinoma occurs much more rarely. This raises the question as to whether a milk factor exists in the human and, if it does exist, whether it is of different kind, acting upon the ducts.

The Differentiation between Prostatic Phosphatase and other Acid Phosphatases in Pathological Human Sera. By FREDA K. HERBERT

In many cases of prostatic carcinoma, especially those with widespread metastases, the specific phosphatase of the prostate is present in the serum. The estimation of total 'acid' phosphatase is a useful diagnostic test, and when high titres are found there is no difficulty in interpretation. In some instances, however, there are only slight increases, such as are occasionally encountered in other diseases, and a method of distinguishing between the prostatic phosphatase and the other acid phosphatases may then be helpful.

Tests based on two distinguishing properties of the prostatic enzyme have been applied to a large number of pathological sera.

(1) The prostatic enzyme is very labile at 37° C. at the pH of serum. Incubation of serum at 37° C. for 1 hr. destroys it. This effect is always demonstrable in prostatic carcinoma with raised serum acid phosphatase, and is absent in the great majority of non-prostatic diseases, but labile phosphatases occasionally occur in non-prostatic diseases and the test is not entirely specific.

(2) The prostatic enzyme is inactivated by alcohols. When serum is allowed to stand $\frac{1}{2}$ hr. at room temperature with four-tenths its volume of ethyl alcohol, the prostatic enzyme is destroyed. This effect is regularly seen in cases of prostatic carcinoma with raised serum acid phosphatase, and is sometimes demonstrable in such cases when the total titre is not definitely raised. In non-prostatic diseases alcohol treatment usually causes no significant change in titre; in some instances there are slight increases or decreases, but never a decrease of more than 1 unit (Gutman and Gutman method, slightly modified). The series of non-prostatic diseases includes a few instances in which the total acid phosphatase was slightly raised, and in these the new method has shown that the enzyme was not of prostatic origin. Further experience is needed, but the alcohol inactivation gives good promise of specificity.

Plasma Phosphatase in the Diagnosis of Malignancy. By E. J. KING and G. E. DELORY

Blood plasma contains two sets of phosphatases, one operating optimally at an alkaline pH, and the other at an acid pH. Small amounts of both alkaline-acting and acid-acting phosphatases are contained in normal blood. Both are measured by the rates at which they hydrolyze phosphoric esters, e.g. phenylphosphate. The King-Armstrong unit of alkaline-phosphatase is that amount of enzyme which liberates 1 mg. of phenol from phenylphosphate at 37° in 30 min. in veronal buffer at pH 9, or in 15 min. in Na_2CO_3-$NaHCO_3$ buffer at pH 10. The unit of acid-phosphatase (suggested by Gutman and adopted here) is the amount of enzyme which liberates 1 mg. phenol from phenyl-phosphate at 37° in 1 hr. in citrate buffer at pH 4·9. Normal plasma contains 5–10 units of alkaline-phosphatase and 1–5 of acid phosphatase. The plasma alkaline-phosphatase

is present in abnormal amounts in cancer of the bone. The work of Franseen,* Woodard,† and Maclagan,‡ and their collaborators, indicates that bony tumours, accompanied by increased osteoblastic activity, show increased plasma phosphatase. Osteogenic sarcoma has a high phosphatase both in bone and in plasma, which is decreased by operation or irradiation. Metastatic carcinoma usually shows an elevated plasma phosphatase. Osteolytic neoplasms, multiple and endothelial (Ewing) myelomas, and giant-cell tumours have normal values.

Abnormal amounts of acid-phosphatase appear as a result of malignant growth in the prostate, or metastatic to it in the skeleton. Kutscher & Wolbergs§ found this highly active acid-phosphatase in prostatic tissue in 1935. Mostly through the work of Gutman‖ and his colleagues this enzyme has been thoroughly studied in the prostate gland, seminal fluid, metastatic growths in bone and the blood plasma. Huggins¶ and co-workers found that prostatic carcinoma as well as adult prostatic epithelium responds to androgenic hormones. Injection of androgen raises the plasma acid-phosphatase, castration and injections of oestrogen cause a fall. Our own work (with Watkinson & Haddow**) amply confirms that of Kutscher, Gutman and Huggins.

Acid-phosphatase is not present in the prostate until puberty. Seminal fluid of normal men contains enormous amounts; that of eunuchoids has very little. Carcinomas of the prostate without bony metastases have had normal plasma acid-phosphatases. With metastases, abnormally high values have been found. The highest value encountered was 108 units. Treatment with stilboestrol has nearly always caused a return of the acid-phosphatase to normal concurrently with improvement of the clinical condition of the patient.

* Franseen, C. C., Simmons, C. S. & McLean, R. (1939). *Surg. Gynec. Obstet.* **68**, 1038.
† Woodard, H. Q. & Higinbotham, N. L. (1941). *J. Amer. med. Ass.* **116**, 1621.
‡ Cade, S., Maclagan, N. F. & Townsend, R. F. (1940). *Lancet*, **1**, 1074.
§ Kutscher, W. & Wolbergs, H. (1935). *Hoppe-Seyl. Z.* **236**, 237.
‖ Gutman, A. B. (1942). *J. Amer. med. Ass.* **120**, 1112; Sullivan, T. J., Gutman, E. B. & Gutman, A. B. (1942). *J. Urol.* **48**, 426.
¶ Huggins, C. (1942). *Ann. Surg.* **115**, 1192; Huggins, C., Scott, W. W. & Hodges, C. V. (1941). *J. Urol.* **46**, 997.
** Watkinson, J. M., Delory, G. E., King, E. J. & Haddow, A. (1944). *Brit. med. J.* **2**, 492.

The Clinical Use of Synthetic Oestrogens in Carcinoma of the Prostate. By E. W. Riches

The treatment of carcinoma of the prostate demands more than the giving of stilboestrol; without adequate investigation and control the results are often unsatisfactory.

Diagnosis, based primarily on rectal examination, may be easy in the scirrus growth, but difficult in the adenomatous type without biopsy. The most effective method of biopsy short of prostatectomy is endoscopic resection of the prostate, and this is also the best method of restoring urinary function in the shortest time. Before starting treatment the general condition, renal function, the presence of metastases, and the level of serum acid phosphatase must be determined. Clinically the predominant symptoms may be:

A. Urinary—difficulty, chronic retention, acute retention.
B. Metastatic—pain, local, referred, or diffuse; oedema.
C. Persistent post-operative suprapubic fistula.

In group A, endoscopic resection procures early relief; it is preceded in cases of retention by suprapubic catheterization. Stilboestrol alone will relieve retention more slowly and less certainly. In group B, resection may not be required. In group C, the fistula will close more quickly after resection than after stilboestrol alone.

In all cases stilboestrol is started as soon as the serum acid phosphatase is known; dose, 1 mg. t.d.s. increased daily by 1 mg. per dose up to 5 mg. t.d.s. or q.i.d. The dose is reduced later according to progress, the level of acid phosphatase serving as a laboratory aid to the clinical picture. Maintenance dose varies from 1 to 10 mg. daily.

Results of stilboestrol therapy. The results to be expected in over 90% of cases are a gain of weight with an improvement in general condition and urinary symptoms; the prostate becomes softer; enlarged lymphatic glands diminish in size and lymphatic oedema disappears. The pain from osseous metastases may disappear. A raised acid phosphatase falls. If the maintenance dose is inadequate the general condition deteriorates and the acid phosphatase rises again.

Side effects. Enlargement of the breasts, pigmentation of the nipples and of the mid-scrotal line, reduction in size of penis and testes, and impotence. Rarely nausea or vomiting ensue but are relieved by changing to hexoestrol or dienoestrol. A patient who pigments deeply responds well.

Failures may be correlated with the histological picture but some apparent failures are due to inadequate dosage. The duration of improvement cannot be foretold; the most striking results are seen in the most advanced cases.

Viruses in Relation to Cancer with Special Reference to the Milk Factor (Afternoon Session)

W. E. Gye. So many ways of inducing cancer have now been discovered that we appear to run the risk of losing sight of the fundamental pathological problem of cancer. If we wish to start a cancer in a laboratory animal we have the choice of perhaps a hundred pure chemical compounds; some very complex and of structures so diverse that it is impossible to correlate structure and effect; some very simple; some related chemically to substances occurring naturally in the body; and some almost as remote as imagination could conceive. Then we have the various forms of radiant energy, gross parasites and viruses. By any of these means we can start a cancer in an appropriate animal, but once the cancer has been started none of the agents used, with the exception of the viruses, are necessary for its continuation. This fact is illustrated in the animal primarily affected by the continued growth of metastases; in laboratory experience by knowledge gained from the grafting of tumours. Tumours which arose in a mouse forty years ago have been grafted in successive groups of animals ever since, and we know that the cells now growing in mice are the lineal descendants of the original cancer cells. All our knowledge supports the view that the cancer cell carries its own stimulus to growth and the stimulus is inexhaustible. This is the essential meaning of autonomous growth and autonomous growth presents the central problem of cancer research. Induction of cancer with chemicals or other agents represents one facet of the problem, and the tendency to separate cancer research into rival groups or compartments will get us nowhere. There is no competition between chemical substances or physical agents with viruses for the honour of causing cancer. Our problem is a jigsaw puzzle, and in the end each part will find a place in the picture.

The pioneers of experimental research recognized that cancer etiology presents two aspects: first, there are the remote causes, which we know are numerous, and which act on cells in ways yet unknown and are not necessary to maintain malignancy; secondly, there is the proximate, intrinsic or, better still, the intracellular cause which provokes the cancer cell to growth and division. Investigators have discovered numerous remote causes; only one intrinsic or intracellular cause has so far been discovered, namely, the cancer viruses. All other explanations of malignant growth have no factual

basis and are purely speculative. The tumours from which an active virus can be extracted are few in number, but not seldom in the history of science has the study of the exception been the key to comparatively rapid advance of knowledge. The immediate practical problem is to reconcile filtrability of some tumours with non-filtrability of others, for there is no doubt that the tumours which can be propagated with cell-free filtrates are the same kind of pathological growth as those which cannot be transmitted with cell-free extracts.

Investigations of filtrable chicken tumours and of Shope's rabbit papilloma and the carcinoma associated with it all support the opinion that the cancer viruses are strictly conditioned in their activities. Moreover, by indirect immunological evidence it has been proved that a virus may be present in a tumour although direct demonstration of it by immediate induction of cancer following inoculation is impossible. Bittner's discovery of the milk factor in breast cancer is a very important additional piece of evidence that a cancer virus can act and produce a cancer only under the most narrowly stringent conditions.

J. McIntosh. I propose to keep my remarks as much as possible along the lines of my personal experience and parallel these to what is known of the milk factor in mammary cancer of mice.

Rous sarcoma no. 1. Investigations on the Rous tumour have shown that it is a true sarcomatous growth with characteristic cellular appearances, typical myxomatous changes, and the formation of metastases. Regarding the causal agent I have demonstrated its particulate nature and its ability to call forth virus-neutralizing and complement-fixing antibodies. These experiments and those of other workers have definitely shown that the Rous tumour agent has antigenic properties comparable to those of bacteria and other viruses.

Induced tumours in fowls. My next step was to determine whether tumours induced in fowls by carcinogenic agents could be shown to contain a virus, and for this purpose tar dissolved in lard was injected into the breast muscle of susceptible Plymouth Rock fowls. In two series of experiments of this nature five tumours were obtained which could, though with considerable difficulty, be transmitted to other fowls by cell-free filtrates. The failure of other workers to confirm these experiments may be due to several factors, but the presence of an agent related to the Rous virus has been demonstrated serologically in some non-filterable induced fowl tumours by Andrewes and other workers.

Shope rabbit papilloma. We have confirmed Rous's findings that the inoculation of domestic rabbits with Shope papilloma virus leads ultimately to carcinoma and that the effects of this virus can be greatly accelerated by previous tarring of the skin. These carcinomata have been transplanted but so far Shope virus has not been recovered from them although serological evidence of the presence of the virus has been obtained—a position analogous to that of some of the non-filterable induced fowl tumours.

The milk factor. The demonstration of the milk agent as the most important factor in determining mammary cancer in mice is one of the most important discoveries in connexion with the aetiology of cancer that has yet come forward and has been fully dealt with by the opener of this discussion. I would, however, like to say that oestrogens in these mice would appear to exert a provocative effect in mammary tissue similar to the role played by tar in the induction of fowl tumours and in the Shope virus experiments.

Conclusion. In conclusion, it would appear that in the aetiology of tumours a parallelism can be drawn between the modes of action of the fowl tumour and Shope viruses and the milk factor as shown in the table. In each case three factors are involved;

the first and most important is the virus, the second is preparation of the tissues by a provocative or localizing agent, and the third is the hereditary susceptibility of the host.

Aetiology of virus tumours

	Causal agent	Provocative agent	Soil
Chicken sarcoma	Virus	Carcinogen tar, etc.	Plymouth Rock
Shope rabbit papilloma	Virus	Carcinogen tar, etc.	Domestic and cotton-tail rabbit
Mouse mammary cancer	Virus (milk factor)	Oestrin	Cancer strain

Dr Mervyn Gordon suggested that current views on the origin of cancer resembled those on tuberculosis before Koch. Following the precept of Harvey 'there is but one road to science, that, to wit, in which we proceed from things more known to things less known', viruses had been approached in the light of knowledge obtained by the study of bacteria. Temperature charts were shown illustrating the improvement effected in acute streptococcal infection by giving a sufficient dose of the combination of antigen with its specific antibodies formed during flocculation by antiserum prepared from rabbits. This improvement was due to the prompt liberation of the antibodies by the tissues on injection.

Investigation during an outbreak of influenza having revealed the presence of vast numbers of elementary bodies in the nasal secretion similar to those present in vaccine lymph, the next step was to study this virus with its unmistakable lesion. Vaccinia was found to evoke the production of specific antibodies in the same way as bacteria. Photographs showing flocculation of vaccinia and variola viruses were shown. Craigie and Tulloch having found that vaccinia virus united with its antibodies during flocculation rapidly produced immunity; this had been confirmed and in addition a therapeutic action observed in the temporary inhibition of vaccinia papules developing on the rabbit's skin. The vaccinia elementary bodies had now been established as the causative organism. Photographs of similar bodies present in the lesions of Hodgkin's disease side by side with those of vaccinia by Coles were shown, and the serum of convalescent cases of Hodgkin's disease yielding evidence of the presence of antibodies, flocculating serum was prepared against them on lines similar to those employed for dealing with the *Streptococcus*. After thorough washing, this combination of the Hodgkin elementary bodies with their antibodies had been tried for treatment by the co-operation of Dr E. C. Warner, and the results had been encouraging. As elementary bodies are present also in the lesions of sarcoma a preliminary trial had been made of the flocculated antigen in that disease also, but the cases were too few for a definite pronouncement at present.

Viruses were a branch of the bacteria and amenable to the same methods of study. Vaccinia was a key to the others. Filtering had the grave defect that it reduced potency—the Seitz disk removed vaccinia virus altogether. Two other avenues of approach confirmed the virus theory of cancer, namely, (*a*) the presence of inclusions in acute cases of carcinoma, and (*b*) an explanation of the relative immunity of the small intestine to new growth by the fact that the fluid contained by it was strongly viricidal owing to the presence of a substance secreted by the pancreas. The action ceased at the large intestine.

C. E. Van Rooyen. In variola there was no difficulty in demonstrating the presence of elementary bodies, as they were very numerous. Employing the same technical methods, smear preparations from lymphadenomatous lymphatic glands occasionally showed minute particles and granules resembling elementary bodies. Unfortunately these were

extremely scanty in numbers, and it was impossible to prove the aetiological relationship of such structures to Hodgkin's disease. Perhaps the use of the electron microscope may be of assistance in the future solution of this particular aspect of malignant disease research.

J. G. Carr. Work upon the two types of carcinogenic viruses, milk factor and fowl sarcoma viruses, has usually proceeded independently, and by differing techniques. It is the purpose of this account to stress certain similarities in their mode of action. While genetic influences upon the action of the milk factor are readily shown by the use of pure line hosts, similar material is not available for the avian sarcoma viruses. But workers in this field have the advantage that the susceptibility of any animal can be immediately ascertained by injecting tumour virus; this is not possible in mice, as tumours arise only a long time after treatment with the virus.

There is a very high incidence of spontaneous tumours in ordinary flocks of fowls—veterinary post-mortem reports give up to 30%—and it is considered that most of these are caused by viruses. It is, therefore, not surprising that such birds are found to be 98–100% susceptible to the Rous no. 1 sarcoma virus. As fowls can act as carriers of tumour virus, such birds are like the high cancer line mice, i.e. susceptible+latent virus. The Brown Leghorn flock raised by Dr Greenwood has an abnormally low tumour incidence—less than 0·1%. These are equally sensitive to Rous 1 virus, and thus appear to resemble fostered cancer mice in being susceptible, but devoid of carcinogenic virus. The susceptibility to Rous no. 1 virus was found to be genetically determined, and Dr Greenwood was able to produce a line of fowls so resistant to this virus that only about 10% produce progressive tumours upon inoculation. These fowls would thus correspond to low cancer strain mice. As in mice the virus can remain latent in such non-susceptible hosts. Maternal transmission of virus from such carriers does not occur in fowls, as it does in mice.

Carcinogenic chemicals will stimulate delayed tumour formation in virus-carrying mice and cause immediate tumour growth in virus-carrying fowls. Low doses of radiation also stimulate tumour production in carrier mice, while Peacock noted that when a Rous tumour was surrounded by radium needles, the tumour disappeared, but in the surrounding tissues, which received a lower dose of radiation, Rous tumours appeared immediately.

Both viruses are disseminated about the tissues of the host. In the fowls, this has been found to be due to the spread of the virus through the vascular system, and is small in amount; comparable studies for mice are not available.

P. R. Peacock. One of the difficulties in any discussion on the etiology of cancer is that pathologists do not all use the word to cover the same group of tumours. Some speak of cancer as synonymous with carcinoma to the exclusion of sarcomata. In view of the fact that a chemical carcinogen (e.g. benzpyrene) will induce carcinoma of the skin or sarcoma of the connective tissues of experimental animals, there is no logical reason for restricting the term 'Cancer' to any one group of malignant tumours. It follows, that in discussing the etiology of cancer the known facts about the causation of any type of malignant tumour must be considered.

It is generally accepted that no infective virus can be demonstrated in most mammalian cancers. Chemical carcinogens that induce various types of cancer in mammals have been used to induce sarcomata in fowls, but have failed to induce carcinoma in this species. The 57 chemically induced fowl sarcomata induced by me resemble the analogous mammalian tumours in all demonstrable respects, and differ from the well-known virus sarcomata of fowls in that they contain no recoverable virus. As most of

the virus tumours originated spontaneously, the initial relationship of the virus to the tumour is not known; it might have been an extrinsic agent or a product of the tumour, though, of course, it becomes the extrinsic cause of experimentally infected fowl virus. McIntosh and Selbie's virus tumour strains are exceptional, but these authors have also reported non-filterable, chemically induced fowl sarcomata. The existence of several types of virus tumours, and even the discovery of further examples in several species of animals, is no logical reason for postulating a viral cause for all tumours against the balance of evidence. There is, in fact, a striking difference between the virus-induced sarcomata and the chemically induced, non-filterable sarcomata of fowls. This difference is the tendency to spontaneous cure in a minority of birds of the filterable tumours—Rous no. 1, Fujinami, and McIntosh V. All these tumours have shown this tendency, in my experience, and Carr has shown that in the case of inbred lines of Brown Leghorns it has a genetic basis. Even more striking is the case of the Fujinami sarcoma in ducks, which regularly undergoes spontaneous cure. As all these virus tumours have been propagated by cell-free filtrates or desiccates, they must be regarded as arising *de novo* at each serial passage from the cells of their hosts. Histologically, these tumours are sarcomata, yet spontaneous cure is certainly not a characteristic feature of sarcoma in the human species. This calls in question the value of histological classification as a guide to etiology. Analogy with other lesions and tissue reaction suggests that etiological deductions based on histology alone would often be erroneous.

A recent review of all the sections of fowl sarcomata in my collection (without knowledge of their origin) showed that several types of sarcoma could be classified with fair accuracy, but no criterion of infectivity could be recognized. Even in the case of virus tumours, recognized as such by their histology, no accurate prediction could be made as to their infectivity. Attention has been drawn recently to this point by Carr, who has shown that the infectivity is in some way related to the age of the tumour. The desire to find a specific cause of cancer probably arises from the idea of cancer as a single disease rather than as a special type of pathological tissue reaction. The weight of evidence available at the present time points to multiple causes of cancer, as in the case of most other types of lesion and tissue reaction.

The **239th Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, W.* 12, *on Saturday,* 3 *February* 1945, *at* 11.15 *a.m., when the following communications and demonstrations were given:*

COMMUNICATIONS

The Microbiological Assay of Pyridoxine. By E. Barton-Wright

The X-ray mutant of the fungus *Neurospora sitophila* (mutant 299) is specific for pyridoxine and can be used for the microbiological assay of this vitamin. The method employed in this investigation is essentially the same as that described by Stokes, Larsen, Woodward & Foster.* It is essential for a successful assay by the *Neurospora* method for (1) all aneurine (B1) must be destroyed, and (2) the pH of the medium must not rise above 5·8. The original medium described by Stokes *et al.* has been slightly modified in two respects: (1) by the addition of sodium dihydrogen citrate (0·2%), and (2) the concentration of zinc sulphate has been doubled. The addition of the sodium dihydrogen citrate slightly stimulates growth and maintains the pH of the medium at 4·6, while the zinc sulphate reduces sporulation to a minimum. Using this method the following values (μg. vitamin B6/g. or ml.) have been obtained for cereals

and other products: whole wheat, 4·3; flour (40% extraction), 1·2; flour (73% extraction), 1·8; flour (85% extraction), 3·1; bran, 13·6, 14·0; germ, 15 5, 17·0; barley, 5·25, 5·9; beer, Reid's stout (brewed 1899), 0·5; Worthington Strong Ale (brewed 1796), 1·2; modern beer, 0·5; dried milk, 8·2; dried meat, 4·7; dried yeast, 15·4.

* Stokes, J. L., Larsen, A., Woodward, C. R. & Foster, J. W. (1943). *J. biol. Chem.* **150**, 17.

The Thymol Turbidity Test of Liver Dysfunction. By N. F. Maclagan

When 3 ml. of a saturated solution of thymol at pH 8, $\mu + 0{\cdot}01$, are added to 0·05 ml. of serum from different individuals a marked turbidity results only in cases where liver damage is certain or probable.* The turbidity is estimated by comparison against urine protein standards and is expressed in arbitrary units, normally 0–4.

Results of the test in 500 cases have been closely parallel to the serum colloidal gold reaction, which is known to depend mainly upon serum γ-globulin.† Phenol and many of its substitution products produce similar effects. The reaction is partly abolished by heating the serum to 37° C. for 30 min., or by ether extraction. Chemical analysis of the precipitate indicates the following average composition: protein 37·5 %, thymol 32 %, cholesterol 16·5 %, phospholipid 8·0 %. It could be redissolved in 0·01-N NaOH and was then completely precipitated by one-third saturation with ammonium sulphate. These findings as a whole suggest that a positive thymol test probably indicates an increased serum γ-globulin content.

Clinical results indicate that the test is of value as a general indicator of liver dysfunction and in the differential diagnosis of jaundice. Thus it was mainly positive in infective hepatitis and cirrhosis (93 %) and was mainly negative in obstructive jaundice (93 %).

* Maclagan, N. F. (1944). *Nature, Lond.*, **154**, 670; (1945). *Brit. J. exp. Path.* (in the Press).
† Kabat, E. A., Hanger, F. M., Moore, D. H. & Landow, H. (1943). *J. Clin. Invest.* **22**, 563.

Photoelectric Determination of Serum Bilirubin. By J. Whidborne and C. H. Gray

Using a Spekker absorptiometer with Ilford 605 spectral filters, the Malloy & Evelyn* method of determination of serum bilirubin is excellent for values above 2·5 mg./100 ml. For values below this, the method is less satisfactory because the final dilution of serum is 1 in 25 giving absorption densities too low for accurate measurement in cells of 1 cm. thickness. It has been impracticable to employ cells of greater thickness. When the technique recommended by McNee & Keefer† is adapted for photoelectric determination, the values obtained are lower than those obtained by the Malloy & Evelyn method, even though the calibration curve is determined by measuring light absorption of azobilirubin solutions adjusted to pH 4·8, i.e. that of the final reaction mixture. It is probable that adsorption on precipitated protein is the cause of this discrepancy.

When the method of Rappaport & Eichhorn,‡ which avoids precipitation of protein, is adapted for the absorptiometer, the results agree well with those obtained by the Malloy & Evelyn procedure, provided azobilirubin solutions adjusted to pH 3·2 are used in determining the calibration curve. Since the final dilution is only 1 in 5 (in our modification 1 in 9) serum bilirubin can be accurately measured when the level is only 0·5 mg./100 ml. or even lower.

Beer's law is followed only for concentrations of azobilirubin pigment corresponding to less than about 1·6 mg. bilirubin per 100 ml. of final solution, i.e. Beer's law is not followed when serum bilirubin is above about 7·0, 8·0, 14·0, and 40·0 mg./100 ml. when

measured by the methods of McNee & Keefer (dilution 1 in 4), original Rappaport & Eichhorn (1 in 5), modified Rappaport & Eichhorn (1 in 9), and Malloy & Evelyn (1 in 25) respectively. If the concentrations are higher than these levels then the final solution should be suitably diluted.

In cases of obstructive jaundice and hepatogenous jaundice, azobilirubin pigment production is maximal within 20 min. in the Rappaport & Eichhorn procedure in the presence of either 'direct' or 'indirect' buffer, but in cases of haemolytic jaundice the production of pigment continues for 5 or 6 hr. even in the presence of the 'indirect' buffer. Unless this fact is taken into consideration the method of Rappaport & Eichhorn cannot determine accurately the percentages of direct and indirect pigments present in the serum or even total bilirubin in cases of haemolytic jaundice.

When the percentage of direct bilirubin is measured by the Rappaport & Eichhorn method the results are higher than when the direct bilirubin is determined by the Malloy & Evelyn method. This is probably due to the difference of pH of the reaction mixtures.§

* Malloy, H. T. & Evelyn, K. A. (1937). *J. biol. Chem.* **119**, 481.
† McNee & Keefer, C. S. (1925). *Brit. med. J.* **2**, 652.
‡ Rappaport, F. & Eichhorn, F. (1943). *Lancet*, **1**, 62.
§ Cabello Ruiz, J. (1943). *Rev. Soc. Argent. Biol.* **19**, 247.

Photoelectric Studies of the Direct Van den Bergh Reaction. By C. H. GRAY and J. WHIDBORNE

The Rappaport & Eichhorn procedure for the determination of 'direct' and 'indirect' bilirubin has been modified for use with a single-cell photoelectric device of the Evelyn type in order to determine the quantity of azobilirubin pigment produced at varying times after the addition of the reagents. It was thus hoped to obtain an accurate representation of the course of the direct Van den Bergh reaction. The curve of azobilirubin pigment production plotted against time was very characteristic in cases of obstructive jaundice, and identical curves were given in hepatogenous jaundice when serum bilirubin exceeded 5 mg./100 ml. whether the Van den Bergh reaction was prompt or biphasic. When the serum bilirubin was below 5 mg./100 ml. the curves remained almost identical but at a slightly lower level; such sera gave delayed direct Van den Bergh responses.

It is possible that the prompt and biphasic responses are due to the fact that Beer's Law is not followed with concentrations of azopigment corresponding to more than about 1·6 mg. of bilirubin per 100 ml. This means that with high concentrations of serum bilirubin, although azobilirubin production follows the usual course, the light absorption and hence the intensity of colour does not increase proportionately and in fact the increase may not be perceptible to the naked eye. Such sera give 'prompt' reactions when investigated by the ordinary technique. With lower levels of serum bilirubin the diminishing rate of increase of light absorption is apparent to the naked eye and such reactions are described as biphasic. In cases of obstructive and hepatogenous jaundice, therefore, the type of direct Van den Bergh reaction depends solely on the concentration of bilirubin in the serum as was suggested by Stein.* The findings of Stein have also been confirmed in that when jaundiced serum of high bilirubin content giving a prompt direct reaction is diluted with normal serum the direct Van den Bergh reaction becomes biphasic or delayed according to the extent of the dilution.

Sera from cases of haemolytic jaundice give quite different curves of azobilirubin production reaching a maximum 6 hr. after the addition of the reagents. There is considerable variation in the exact form of these curves; in some, true biphasic curves have been observed with an abrupt decrease in slope during the course of the reaction. The significance of these curves remains obscure.

* Stein, H. B. (1941). *S. Afr. J. Med. Soc.* **6**, 104.

Cholic Acid Tolerance in Liver Diseases. By S. SHERLOCK and V. WALSHE

An Iodimetric Method for Plasma Bicarbonate. By E. J. KING and I. D. P. WOOTTON

The following reactions have been utilized to determine the total CO_2 of Na_2CO_3 solutions, $NaHCO_3$ solutions and blood plasma:

$$CO_2 + Ba(OH)_2 \rightarrow H_2O + BaCO_3\downarrow,$$
$$Ba(OH)_2 + 2NaIO_3 \rightarrow 2NaOH + Ba(IO_3)_2\downarrow,$$
$$Ba(IO_3)_2 + 12HI \rightarrow 6I_2 + BaI_2 + 6H_2O,$$
$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI.$$

The CO_2-containing solution is treated in a conical centrifuge tube with a measured excess of barium hydroxide solution. The CO_2 is quantitatively precipitated. The excess $Ba(OH)_2$ is then precipitated by adding sodium iodate. The combined precipitate of $BaCO_3$ and $Ba(IO_3)_2$ is spun down, washed and dissolved in acid. Potassium iodide is added and the liberated iodine titrated with thiosulphate. A blank is carried through at the same time, using the same amount of barium hydroxide and no carbonate or bicarbonate. The difference between the titration of the blank, in which all the barium was precipitated as $Ba(IO_3)$, and that of the test, gives the equivalent in terms of iodine, of the barium which was precipitated as $BaCO_3$. From the above equations, 12 ml. of N-iodine are equivalent to 22·4 ml. of CO_2.

Production of the Antibacterial Protein Diplococcin in a Medium Containing no Protein or Polypeptide. By A. E. OXFORD

It was recently shown* that certain lactic streptococci, grown in a simplified medium containing as little as 5 % of heart broth for the provision of growth factors and essential amino-acids, produced an antibacterial protein, diplococcin, which could be extracted from the bacterial cells. It has now been conclusively demonstrated that diplococcin is built up from its constituent amino-acids by the micro-organism, rather than derived from complete protein or polypeptide molecules supplied in the medium. The medium used for this purpose was Niven's simplified protein- and polypeptide-free medium devised for the growth of *Str. lactis*,† which contains nothing more complex than a total acid hydrolysate of casein (0·5 %) together with salts, growth factors, and a carbohydrate, in this instance sucrose (0·75 %). The concentration of K_2HPO_4 was increased to 0·8 % in order that growth might be prolonged so that relatively much diplococcin appeared in the medium. It may be extracted from the resulting metabolism solution in the form of an acid-soluble protein, by removal of the cells, evaporation of the clear supernatant to small volume below 60° *in vacuo*, and extraction of the precipitate so formed, easily removable by centrifuging, with cold very dilute acetic acid. It cannot be extracted from this precipitate in the cold at pH 7. The active protein, which is far more potent than any previous preparation from the bacterial cells, may be precipitated from the acidic extract by 60 % saturation with ammonium sulphate. The bacterial cells grown in Niven's medium will also yield crude diplococcin by the old procedure, but the preparation so obtained is heavily contaminated with inert protein.

* Oxford, A. E. (1944). *Biochem. J.* **38**, 178.
† Niven, C. F. (1944). *J. Bact.* **47**, 343.

A New Type of Phase Rule Solubility Test for Enzyme Purity. By J. S. FALCONER and D. B. TAYLOR

On the Absence of a Connexion between the Growth Retardation Produced by Polycyclic Aromatic Hydrocarbons and Sulphur Metabolism. By F. L. WARREN, L. A. ELSON and F. GOULDEN

White *et al.** concluded that the retardation of growth produced in young rats by administration of such polycyclic aromatic hydrocarbons as 3:4-benzpyrene, methylcholanthrene, or pyrene, was due to deprivation of sulphur-containing amino-acids. They assumed that such sulphur-containing acids were necessary for detoxication by mercapturic-acid formation.

To test this hypothesis we have examined the urinary sulphur partition in rats injected with benzene, naphthalene, phenanthrene, anthracene, 1:2-benzanthracene, 1:2:5:6-dibenzanthracene, 3:4-benzpyrene, pyrene, or chrysene. The effects of these hydrocarbons on the growth rates were also observed.

No evidence of the excretion of mercapturic acids by rats treated with the carcinogens 3:4-benzpyrene and 1:2:5:6-dibenzanthracene was found. The retardation of growth (amounting to complete cessation in these experiments) produced by these hydrocarbons cannot be attributed to a deprivation of sulphur-containing amino-acids.

This conclusion is contrary to that reached by White and his colleagues. They base their conclusions on the restoration of growth observed on feeding cystine or methionine to rats retarded in growth by the inclusion of hydrocarbons in their diet. Rats on the supplemented diet, but still ingesting hydrocarbon, showed the same growth rate as those eating an unsupplemented diet free from hydrocarbon.

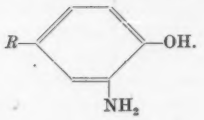
Criticism of this method as used by White *et al.* may be summarized by pointing out that the rats receiving the supplemented diet plus hydrocarbon would be expected to show a *higher* growth rate than rats on the unsupplemented diet free from hydrocarbon, owing to the superior nutritive value of the supplemented diet.

Experiments carried out by the present authors with the diets used by White *et al.* bear out this expectation. The experiments described by White *et al.* cannot, therefore, be considered to show proof of a connexion between the growth retardation produced by polycyclic aromatic hydrocarbons and sulphur metabolism.

* Cf. e.g. *J. biol. Chem.* **131**, 149 (1939).

Antibacterial Activity of 2-Aminophenols. By M. BARBER and G. A. D. HASLEWOOD

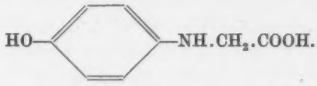
In a preliminary report,* it was stated that interest might attach to the preparation and testing for antibacterial activity, especially against Gram-negative organisms, of a series



We have now made and tested compounds in which

R = —CH_3, —CH_2CH_3, —$(CH_2)_2CH_3$, —$CH(CH_3)_2$, —$(CH_2)_3CH_3$, —$C(CH_3)_3$, —$CO.CH_3$, —$CO.CH_2.CH_3$, and —$CO.(CH_2)_2CH_3$.

Activity of the same order as that of 2-aminophenol itself was shown by the methyl, ethyl, and *n*-butyl compounds; the remainder were less active. No substances with oxygen in the side-chain have shown high activity. There are indications that antibacterial properties are not confined in these aminophenols to primary amines: they were shown, for example, by 'metol' and by *p*-hydroxyphenyl glycine,



The antibacterial activity of 2-aminophenols depends *inter alia* on the molecular structure and state of oxidation of these substances: the most obviously unstable (easily oxidized) compounds are not necessarily antibacterial. Activity *in vitro* under our conditions of testing was abolished by ascorbic acid in molecular concentration about one-third of that of the aminophenol.

The investigation is being continued.

* Barber, M. & Haslewood, G. A. D. (1944). *Brit. med. J.* **2**, 754.

The Nature of Ascorbic Acid Oxidase. (1) A Critique of the Copper-Protein Theory. By L. H. Lampitt and D. H. F. Clayson

When a solution containing $Ca(HCO_3)_2$, an alkali phosphate and a trace of ionized copper, is heated to about 55° C., its catalytic activity against ascorbic acid is destroyed or considerably reduced.* The unheated mixture may therefore be regarded as a synthesized ascorbic acid oxidase. As the components of this system are present in living cells, the possibilities have been considered that (*a*) the synthesized ascorbic acid oxidase is identical with that occurring naturally, (*b*) oxidation of ascorbic acid in the living cell is wholly or partially due to Cu^{++}.

The latter consideration admittedly reopens an old controversy. The copper-protein theory depends mainly on the constancy of the relationship between Cu^{++} and activity. There is at present, however, no clear spectrographic or other evidence as to the function of the protein of the complexes isolated by various workers in attempts to purify ascorbic acid oxidase. If the copper of these complexes becomes partially ionized in aqueous solution, as is probably the case, the catalytic activity will depend on the degree of ionization, unless the protein fraction itself accelerates or retards secondary reactions. Stotz, Harrer & King† found that the catalytic effects of ascorbic acid oxidase preparations from natural sources and of artificial copper-protein complexes were less than those of amounts of Cu^{++} equal to those present in the complexes. Straub‡ disputed these results on account of limited data, showing that the activities of the redissolved ashes of cucumber preparations were less than those of the preparations themselves. His technique, however, was inadequately described, e.g. the temperature of ashing was not mentioned and copper checks were not reported. Even with low temperature ashing, silica can make copper virtually insoluble in acid unless removed by HF. Experiments with the necessary refinements in technique are in progress to prove or disprove Straub's results.

As calcium phosphate is considered to be present in the form of loose complexes with proteins in biological fluids such as serum and milk and also in plant cells, copper may exist in an environment comparable with that of synthesized ascorbic acid oxidase and yet be associated with protein.

* Lampitt, L. H., Clayson, D. H. F. & Barnes, E. M. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 193.
† Stotz, E., Harrer, C. J. & King, C. G. (1937). *J. biol. Chem.* **119**, 611.
‡ Straub, F. B. (1938). *Hoppe-Seyl. Z.* **254**, 203.

The Nature of Ascorbic Acid Oxidase. (2) The Reactions between Ionized Copper, Potassium Phosphate and Calcium Phosphate. By L. H. LAMPITT, D. H. F. CLAYSON and E. M. BARNES

In order to have a background from which to assess the effects of Cu^{++} in biological systems, a study has been made of the solubility of copper in the presence of phosphate under various conditions. It is appreciably soluble in the presence of an excess of potassium phosphate, presumably because of the formation of double salts (cf. Bassett & Bidwell*). The solubility decreases rapidly with increase of pH up to 9·0, but under a wide range of conditions Cu^{++} concentrations are sufficient to produce pronounced catalytic effects on ascorbic acid. Calcium has little effect on the solubility of copper in potassium phosphate below pH 7·0, but above this pH small Ca^{++} concentrations (e.g. 6×10^{-5} M) reduce copper solubility considerably, and at pH 8·75 copper can be almost completely eliminated ($< 1 \times 10^{-5}$ M).

Fixation of Cu^{++} on $Ca_3(PO_4)_2$ gel is accompanied by liberation of Ca^{++}; the reaction may therefore be considered to be of the base exchange type. Cu^{++} can be redissolved from this complex by potassium phosphate at rates depending on the concentration of pH of the latter. With 0·5 M-K_2HPO_4 the rate of solution was found to be similar to the rate elution of polyphenol oxidase from $Ca_3(PO_4)_2$ adsorbent by this solution as described by Keilin & Mann.† The purification process developed by these authors, which formed the basis of similar processes for purifying ascorbic acid oxidases developed by other workers, does not therefore differentiate between Cu^{++} and copper-protein. Moreover, the reagents used were potential sources of extraneous Cu^{++}.

$Ca_3(PO_4)_2$ gel containing Cu^{++} can be brought into solution by passing in CO_2, and reprecipitated on removing CO_2 by heat, aeration or by the addition of solvents, such as acetone, that reduce the solubility of CO_2 in water.

Reprecipitation is accompanied by loss of catalytic activity. In order to determine whether similar reactions occurred in cabbage juice, the juice was (*a*) aerated in a stream of CO_2-free air, (*b*) concentrated to half its volume under reduced pressure at below 40° C. to avoid protein denaturation. The catalytic activity of the juice was unaffected, in spite of liberation of CO_2. If this activity be due to $Cu^{=}$, prevention of precipitation of Cu^{++} must be attributed to some factor other than HCO_3. Organic acids would be anticipated to have this effect, particularly citric acid. The presence of this acid in cabbage juice has been confirmed, and its regulatory effect on ascorbic acid oxidation is at present being studied.

* Bassett, H. & Bidwell, W. L. (1933). *J. chem. Soc.* pp. 854, 871, 877.

† Keilin, D. & Mann, T. (1938). *Proc. Roy. Soc.* B, **125**, 187.

A Rapid Colorimetric Method for Cereal Proteinase. By R. GORDON BOOTH and J. B. HUTCHINSON

Extracts from finely ground cereal samples are made by the use of a suitable protein dispersant. Weak acetate buffer + NaCl and a preservative (merthiolate) has been found most efficient for this purpose, leaving very little residual proteinase activity in the sample. The native cereal protein extracted with the enzyme is very insensitive to proteolytic attack and variable quantities and qualities present have no bearing on the method. A 1 % urea-denatured haemoglobin substrate buffered at pH 5·0 (similar in principle to that used by Anson*) is employed, and the production of tyrosine + tryptophane from this substrate under standard conditions of time and temperature is measured by the use of the Folin-Ciocalteu reagent.

Tyrosinase does not interfere with the estimation, as has been shown by complete recovery of tyrosine added to and incubated with the enzyme-substrate mixture.

* Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.

The Estimation of Ascorbic Acid for Clinical Purposes by the Hydrazine Method.

By B. B. Lloyd, H. M. Sinclair and G. R. Webster

Roe & Kuether* determined ascorbic acid in whole blood or urine by oxidizing it and estimating colorimetrically a dinitrophenylhydrazine derivative in acid solution. This method also estimates any dehydroascorbic acid or 2:3-diketogulonic acid present in the blood or urine, but the authors showed that other substances, such as glucose or pyruvate, did not normally interfere. One advantage of the method is that comparatively old samples of blood or urine, in which appreciable oxidation of ascorbic acid may have occurred (though not beyond the diketogulonic stage), may successfully be estimated.

During nutritional surveys in the last 15 months some 8000 routine estimations have been done with Roe's method by one of us (B.B.Ll.) on urine, on whole blood and on all parts of blood; and initially the plasma and leucocyte-platelet estimations were duplicated by estimations done by the indophenol method using photoelectric colorimetry. 611 samples of human plasma have given the following results, expressed as mg. ascorbic acid per 100 ml.:

No. of samples: 611.

Mean $\pm$ S.E.:	Indophenol $0{\cdot}570 \pm 0{\cdot}016$; Hydrazine $0{\cdot}741 \pm 0{\cdot}018$
Range:	Indophenol 0·06 $-1{\cdot}52$; Hydrazine 0·07 $-1{\cdot}89$
Correlation coefficient $\pm$ S.E.:	$0{\cdot}950 \pm 0{\cdot}004$
Linear regression lines:	Hydrazine $= 1{\cdot}088$ (indophenol $+ 0{\cdot}121$)
	Indophenol $= 0{\cdot}829$ (hydrazine $- 0{\cdot}044$)

There is therefore a highly significant correlation between estimations done by the two methods, but those done by the hydrazine method are generally 0·1 to 0·3 mg./100 ml. higher. This might be due either to the indophenol method giving low results owing to oxidation of ascorbic acid before estimation, or to the hydrazine method giving high values owing to the inclusion of dehydroascorbic and diketogulonic acids; Penney & Zilva† found no ascorbic acid or dehydroascorbic acid, and about 5·4 mg. diketogulonic acid per 100 ml. plasma of guinea-pigs fed for 5 days on a scorbutic diet; this very high value does not agree with Roe's original figures for whole blood in scorbutic guinea-pigs or with figures obtained by us for whole blood in guinea-pigs on controlled diets. The former explanation seems more probable as about 1 hr. elapsed between venepuncture and metaphosphoric acid precipitation for the indophenol plasma estimations.

The amount of ascorbic acid in plasma appears to reflect the immediate past intake of the vitamin, but the leucocyte-platelet layer of centrifuged blood is relatively less affected and therefore estimations of ascorbic acid in it provide better information for clinical purposes. We have therefore included this estimation in nutritional surveys since 1941. The estimation is troublesome if done by the indophenol method because the leucocyte-platelet layer is often inseparably mixed with a small percentage of haemoglobin which appears to oxidize ascorbic acid at the moment of precipitation of proteins with metaphosphoric acid, giving low values; we have therefore routinely discarded results obtained from 'pink' leucocyte-platelet layers. This objection does not apply to the hydrazine method. Results obtained by the two methods upon the leucocyte-platelet layer of human blood samples are as follows (values expressed as mg. ascorbic acid per 100 g. leucocyte-platelet layer):

No. of samples = 89.

Mean $\pm$ S.E.:	Indophenol $5{\cdot}82 \pm 0{\cdot}33$; Hydrazine $11{\cdot}15 \pm 0{\cdot}39$
Range:	Indophenol 0·0 $-15{\cdot}4$; Hydrazine 2·8 $-22{\cdot}7$
Correlation coefficient $\pm$ S.E.:	$0{\cdot}599 \pm 0{\cdot}071$
Linear regression lines:	Indophenol $= 0{\cdot}505$ (hydrazine $+ 0{\cdot}187$)
	Hydrazine $= 0{\cdot}709$ (indophenol $+ 7{\cdot}029$)

The correlation, though significant, is not as good as that obtained with plasma probably because of low values being obtained by the indophenol method owing to oxidation in the leucocyte-platelet layer before precipitation, which often could not be effected before 12 hr. after venepuncture.

The indophenol method cannot be used for estimation of ascorbic acid in whole blood or in erythrocytes unless special and tedious precautions are taken to exclude oxygen during precipitation of proteins; the hydrazine method is, however, very convenient for these estimations. In 710 samples of human blood estimations have been made by this method upon the whole blood and its separated fractions; i.e. plasma, leucocyte-platelet layer, and erythrocytes; and the value for whole blood has also been calculated from the estimations on the three fractions of blood and their observed relative volumes. The following results compare the observed and calculated amounts of ascorbic acid in whole blood (values expressed as mg. ascorbic acid per 100 ml. blood):

No. of samples = 710.	
Mean ± S.E.:	Observed 0·755 ± 0·014; Calculated 0·736 ± 0·012
Range:	Observed 0·07 − 1·83; Calculated 0·09 − 1·84
Correlation coefficient ± S.E.:	0·970 ± 0·002
Linear regression lines:	Observed = 0·9925 (calculated + 0·025)
	Calculated = 0·949 (observed + 0·020)

Comparisons have also been made, using the hydrazine method, between the concentrations of ascorbic acid in the different parts of blood and between these concentrations and the change in amount excreted in urine before and after administering the vitamin by mouth. These results will be communicated later.

We conclude that the hydrazine method for the estimation of ascorbic acid in blood or the different parts of blood, is more convenient than the indophenol method, and is reliable for clinical purposes or nutritional surveys.

* Roe, J. H. & Kuether, C. A. (1943). *J. biol. Chem.* **147**, 399.
† Penney, J. R. & Zilva, S. S. (1943). *Biochem. J.* **37**, 403.

Comparison of Methods of Estimating Blood Proteins. By R. H. A. Plimmer and L. Lowy

The micro-Kjeldahl, Nessler, tyrosine and biuret methods have given results agreeing within ± 0·4 g./100 ml. The salicylsulphonic acid photometric method has not given satisfactory agreement.

Some slight modifications have been made in the usual procedures. In the micro-Kjeldahl method: as the albumin in the sodium sulphite filtrate is not completely precipitated by zinc sulphate and sodium hydroxide, it is precipitated by sodium tungstate, or preferably sodium molybdate, and sulphuric acid. In the tyrosine and biuret methods: the globulin is removed by half saturation with ammonium sulphate and the albumin is precipitated with sodium tungstate and sulphuric acid. The albumin precipitate dissolves quickly in sodium hydroxide solution and is ready for further treatment.

DEMONSTRATIONS

The Estimation of Cholic Acid in Blood. By V. WALSHE

A $NaHCO_3$-Na_2CO_3 Buffer for Studying Alkaline Phosphatases. By E. J. KING and G. E. DELORY

Field Methods for the Estimation of Mepacrine; Excretion Products of Mepacrine in the Urine. By E. J. KING and M. GILCHRIST

Comparison of Methods of Estimating Blood Proteins. By R. H. A. PLIMMER and L. LOWY

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held at the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1, on Saturday, 17 March* 1945, *at 2 p.m., when the following papers were read:*

COMMUNICATIONS

Effect of Advancing Age on the Assimilation of Calcium by the Rat. By K. M. Henry and S. K. Kon

The Ca retention of male rats from diets containing varying amounts of Ca and P was determined at intervals between the ages of 1 month and 2 years. Diet 191 consisted of dried coagulated egg-white 13·5%, sugar 9%, maize starch 64·8%, fat 10%, Ca- and P-free salts 2·7%, supplemented with pure vitamins. Diet 193 contained 89% of diet 191, 10% of dried skim milk and 1% of fat. Ca and P were only present as impurities in diet 191; the P content of the other diets was adjusted by the addition of Na_2HPO_4, $12H_2O$. The animals received approximately 5 i.u. vitamin D daily. Between metabolic experiments they were given our stock diet.* Two groups of rats were used; in each group comparisons were between litter mates and initially each diet was given to 6 rats. The % Ca retentions are shown below.

Approximate age of rats	Diet 191 0·011% Ca 0·017% P Group 1	Diet 198 0·011% Ca 0·030% P Group 2	Diet 192 0·011% Ca 0·059% P Group 1	Diet 192 0·011% Ca 0·059% P Group 2	Diet 199 0·011% Ca 0·113% P Group 2	Diet 193 0·129% Ca 0·229% P Group 1
1 month	−345	−193	− 20	+ 41	+ 63	+96
6 months	−119	−129	−121	− 81	− 84	+22
1 year	−256	−217	−169	−245	−219	+10
2 years	−409	−192	−244	−196	−201	− 7

Except with diets containing insufficient Ca and P to cover the minimum requirements of the animals† Ca retention usually decreased progressively with advancing age and with all diets there was a gradual increase of faecal Ca. The urinary Ca increased consistently only with diet 193 and to a lesser extent with diet 199. A decreased Ca retention was to be expected as skeletal growth ceased, but it is noteworthy that at 2 years rats were actually losing body Ca with a diet from which they retained 96% Ca when young.

At the end of these tests the retention of Ca from our stock diet* (0·46% Ca, 0·59% P) was determined on the surviving animals in each group; the retention was +1% in group 1 and +3% in group 2. Subsequently group 2 was given a full human diet‡ of lower Ca content (0·119% Ca, 0·221% P) and the Ca balance again became negative (−17%). This negative balance persisted for a month and was doubtless due to a loss of Ca from the bones and not to elimination of any mobile reserves.

It is obvious that to maintain Ca equilibrium in senescence rats require a higher concentration of dietary Ca than in early maturity. These findings underline the need for further consideration of the demineralization of the skeleton known to occur in old age in man.

* Folley, S. J., Ikin, E. W., Kon, S. K. & Scott Watson, H. M. (1938). *Biochem. J.* **32**, 1988.
† Henry, K. M. & Kon, S. K. (1939). *Biochem. J.* **33**, 173.
‡ Macrae, T. F., Henry, K. M. & Kon, S. K. (1943). *Biochem. J.* **37**, 225.

Thymol and Phosphatase Tests in Jaundice. By N. F. MACLAGAN

The thymol turbidity test* and the serum alkaline phosphatase estimation† have been carried out in 114 cases of jaundice in which the diagnosis was subsequently verified by operation, autopsy, or unequivocal clinical data. Normal limits for these two tests are 0–4 and 3–13 units respectively. In all cases with a normal thymol test and a phosphatase above 35 units jaundice was due to mechanical obstruction of the biliary tract (23 cases). All cases with a thymol test above 7 units (42 cases), or with a thymol test of 5–7 units and in addition a phosphatase below 36 units, were non-obstructive (16 cases). Other combinations of results (33 cases) occurred in both groups and were of no diagnostic value.

The results as a whole suggest that a combination of these two tests promises to distinguish obstructive from non-obstructive jaundice in about 71 % of cases.

* Maclagan, N. F. (1944). *Nature, Lond.*, **154**, 670. (1945). *Brit. J. Exp. Path.* (in the Press).
† King, E. J. & Armstrong, A. R. (1934). *Canad. Med. Ass. J.* **31**, 376.

Nutritive Value of the Nitrogenous Substances Contained in a Watery Beef Extract. By H. CHICK and E. B. SLACK

Beef extract, prepared according to a domestic recipe for 'Beef Tea', was included as sole source of nitrogen in an otherwise complete diet consisting of pure maize starch, arachis oil and salt mixture, with cod-liver oil and a protein-free yeast extract to provide, respectively, vitamins A and D and the B group of vitamins. Newly weaned rats receiving this diet showed no weight increase even when the N was raised to 3·6 %, equivalent to over 20 % 'crude protein' in the diet. When, however, the meat extract provided one-quarter of the N in the diet and three-quarters was derived from white flour, the rats greatly outstripped in growth littermates on a diet containing the same amount of N (2·1 %, or 12·5 % protein), provided solely by white flour; the average final body weights at the end of 12 weeks were 227 and 154 g. respectively. The protein in these diets was suboptimal in amount and was the factor limiting growth.

The rats on the beef tea-flour diet ate more food than those on the flour diet and made better use of it, as measured by g. dry food ingested per g. weight increase (4·6 and 6·6 respectively) and also made better utilization of the N, as measured by g. weight increase per g. N ingested (10 and 7·4 respectively).

In the beef tea 70 % of the N was present as gelatine. A diet similar to the above in which one-quarter of the N was derived from pure gelatine and three-quarters from white flour, restored growth to rats which had failed on beef tea N alone, at a rate equal to that developed on the beef tea-flour diet (average body-weight increase per rat per week 15·7 and 15·3 g. respectively).

The nutritive value of the nitrogenous substances in beef tea can therefore be attributed mainly to the gelatine it contains. Their supplementary value for those of white flour in maintaining growth is probably due to the high content in gelatine of lysine, an amino-acid essential for growth which is present in cereal proteins in relatively small amount.

A Simplified Plasma Coagulum for Tissue Cultures. By C. WAYMOUTH and J. N. DAVIDSON

It is the usual practice in tissue culture technique to allow explanted tissues to grow in a fibrin coagulum prepared by allowing whole plasma to clot. This type of coagulum has physical properties ideal for the purpose, but fluid plasma cannot be stored for more

than a few weeks and has the disadvantage for quantitative work of being complex and variable in composition. In connexion with experiments on the influence of phospholipins on tissue growth, it has been necessary to devise a medium as free from lipins as possible, and for this purpose fowl plasma proteins freed from lipoid material by the method of Hewitt* (treatment with 7:3-ethanol-ether mixture at about $-10°$ followed by extraction with ether) have proved suitable. This medium resembles in many respects Fischer's† dialyzed plasma but has the advantage of stability and ease of storage.

The plasma protein powder may be sterilized by allowing it to stand under benzene or ether for several days, but it is best prepared aseptically. Blood is drawn from the carotid artery of a fowl into ice-cold sterile waxed centrifuge tubes and the plasma so obtained is defatted by Hewitt's method with aseptic manipulation using sterile glassware. The dry plasma protein powder is weighed out into sterile tubes and dissolved as required in sterile Tyrode solution. The solution of redissolved protein is alkaline (about pH 8) and is adjusted to pH 7·4 with N/50-HCl.

The plasma protein solution contains sufficient prothrombin for clotting to occur readily when tissue fragments are added to it.

* Hewitt, L. F. (1927). *Biochem. J.* **21**, 216.
† Fischer, A. (1941). *Acta physiol. scand.* **2**, 143.

Factors in Aqueous Extracts of Peas Responsible for Penicillin Production.

By R. P. Cook, W. J. Tulloch, Margaret B. Brown and J. Brodie

Hot-water extracts of freshly ground dried peas form a good medium for the production of penicillin.* Experiments have been carried out to determine what constituents are responsible for the effect. The preparation and fractionation is described in the succeeding communication. Hot- and cold-water extracts have been compared.

The basic medium consisted of 'nutrient' salts as in the Coghill formula, 1% (w/v) NaCl† and 3% (w/v) lactose. The fractions from the pea extract were initially added in the proportions as found in a '10% extract'.

The *P. notatum* (1249 B21) was grown in 40 ml. of medium in 250 ml. conical flasks at 24°. Penicillin titres were determined by Brodie's method.‡ Typical results are presented in the table.

		Dry wt. fraction g./100 ml.	N g./100 ml.	Penicillin production units/ml. ( ) = days	Wet wt. pressed felt g.	pH ( ) = days
IV. Clarified extract	Hot ext.	2·00	0·086	225 (12)	1·92	8·4 (14)
	Cold ext.	2·21	0·113	200 (8)	2·78	7·6 (10)
V. Resuspended 80% alcohol ppt.	Hot ext.	1·02	0·052	200 (12)	2·03	7·9 (14)
	Cold ext.	0·78	0·029	100 (10)	1·47	7·3 (10)
VI. 80% alcohol-soluble material	Hot ext.	0·82	0·025	10 (12)	1·27	7·4 (14)
	Cold ext.	1·54	0·058	25 (10)	2·31	7·4 (10)
V + VI	Hot ext.	1·83	0·077	200 (12)	1·99	8·5 (14)
V + VI	Cold ext.	2·32	0·087	200 (8)	1·97	7·6 (10)

With cold-water extracts 'covering' is more rapid and penicillin production reaches a peak earlier. Further fractionation indicates that the proteose portion of fraction V is not essential. The ash of fraction V in combination with fraction VI (hot-water extracts) gives yields of 100 units/ml. The amount of fraction V added and the yield of penicillin are independent over a wide range.

* Cook, R. P. & Tulloch, W. J. (1944). *J. Path. Bact.* (in the Press).
† Tomkins, R. G. Unpublished observations.
‡ Brodie, J. (1944). In the Press.

Some Constituents of Aqueous Extracts of Ground Dried Peas. By R. P. Cook and Margaret B. Brown

Hot or cold aqueous extracts of freshly ground air-dried peas were fractionated:

100 g. pea meal extracted repeatedly with tap water and pressed through muslin

I. Unclarified extract. Volume made up to 1 litre '10% extract'

II. Residue.

Centrifuged

IV. Clarified extract. Four times volume ethyl alcohol added

III. Clarification residue

V. 80% alcohol ppt. Resuspended in water

VI. 80% alcohol soluble material. Made up to volume after removal of alcohol.

An average analysis of some of these fractions is presented. There is some variation of composition, particularly in the clarified extract in different batches. The results are expressed as (*a*) percentage of the original pea meal and (*b*) percentage of the clarified extract (IV).

The pea meal contained ash 3·38 g. % and total N (Kjeldahl) 3·3 g. %.

		Dry wt.		Ash		Total N		N.P.N. (N not pptd by trichloracetic acid)
		a	*b*	*a*	*b*	*a*	*b*	*b*
II. Residue after extraction	Hot ext.	60	—	32	—	47	—	—
	Cold ext.	50	—	19	—	32	—	—
III. Clarification residue	Hot ext.	20	—	12	—	17	—	—
	Cold ext.	26	—	3	—	34	—	—
IV. Clarified extract	Hot ext.	20	100	54	100	27	100	100 [67% of total N]
	Cold ext.	23	100	78	100	34	100	100 [80% of total N]
V. 80% alcohol ppt. resuspended	Hot ext.	10	50	44	83	17	64	56
	Cold ext.	8	35	45	57	11	31	32
VI. 80% alcohol-soluble material	Hot ext.	10	50	9	17	8	30	44
	Cold ext.	15	65	30	36	14	42	68

The following general conclusions may be drawn. Cold-water extracts remove more soluble material containing a higher percentage of the total ash and total N. A large proportion of the ashable material is precipitated by 80% alcohol, particularly with hot-water extracts. The resuspended alcohol precipitate may be further fractionated by removing insoluble protein and polysaccharides. The water-soluble portion on drying contains ash 15% carbohydrate (as glucose after acid hydrolysis) 30%, N 4·3%, and a portion soluble in ether after acidification.

Acid Phosphatase of the Red Cells. By E. J. King, E. J. Wood and G. E. Delory

In confirmation of the work of Behrendt,* a phosphatase has been found in washed human red blood cells. It is extremely active against the phosphoric esters of phenols but shows very little activity against phosphoric esters of alcohols, glycerol and hexoses. With phenylphosphate as substrate the enzyme is optimally active between pH 4·8 and 5·2. It is much less active at pH 4 and 6, and has only slight activity at pH 7. It is inhibited by Mg, F and long treatment by ethanol, it is activated by Mn and CN; and is almost unaffected by glycine, ascorbic acid and sulphanilamide. The amount of the enzyme has been estimated in the red cells of a variety of human subjects. The cells are diluted and laked with distilled water (1 in 10); 0·2 ml. of this solution (=0·02 ml. of red cells) is added to 2 ml. of buffer (pH 4·9) and 2 ml. of M/100 sodium phenylphosphate; and the mixture is kept at 37° for 30 min., when the enzyme

action is stopped by the addition of 1·8 ml. of dilute (1 in 3) Folin and Ciocalteu phenol reagent. To a control the phenol reagent is added immediately after mixing the red cell solution and substrate. The liberated phenol is determined colorimetrically against a suitable standard by adding 1 ml. of 25% Na_2CO_3 to 4 ml. of supernatant from test and control after the precipitated protein has been spun off. Results are expressed in units equal to mg. phenol hydrolyzed per 100 ml. of red cells. The blood of normal persons had from 200 to 400 units with an average of 340. No abnormalities in the amounts found in disease have thus far been observed.

* Behrendt, H. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 268.

Seasonal Changes in True and Apparent Vitamin C in Walnuts. By F. Wokes, R. Melville, J. G. Organ and E. M. James

True and apparent vitamin C have been estimated by methods previously described in different tissues of *Juglans regia* and allied species at intervals during the summer, autumn and winter. In the nuts during growth and maturation there is a gradual decrease in the concentration of apparent vitamin C, accompanied at first by an increase in the concentration of true vitamin C. The apparent vitamin C occurs mainly in the outer tissues (epicarp and mesocarp) and reaches a concentration (expressed as equivalent to ascorbic acid in dye-reducing power) of 1500 mg./100 g. in nuts less than 1 g. weight. Very little is found in the endocarp, which contains the highest concentration of true vitamin C. Statistical examination of the results on *J. regia* shows high significant negative correlation between apparent and true in epicarp, and significant positive correlations between apparent in mesocarp and true in endocarp, and true in epicarp and endocarp respectively. There is also a high significant positive correlation between apparent in epicarp and true occurring 14 days later in endocarp. This time lag may be related to translocation into the endocarp from the epicarp or other centres of photosynthetic activity, especially the leaf mesophyll, where apparent vitamin C occurs in high concentration, up to 500 mg./100 g. The highest concentrations of apparent are found in the buds and male catkins (in the latter of which it may exceed 2000 mg./100 g.) and appear to remain at a high level throughout autumn and winter. Considerable amounts of apparent vitamin C are present also throughout summer, autumn and winter in the conducting tissues (particularly the phloem) of the branches. These findings are in general conformity with the hypothesis that apparent vitamin C is a precursor of true vitamin C in the walnut.

The Densitometric Estimation of Total Serum Protein. By B. B. Lloyd, E. B. Cheek, H. M. Sinclair and G. R. Webster

The density-protein relation in serum has been investigated by Moore & Van Slyke* using humans (mainly nephritic), and Weech, Reeves & Goettsch† using dogs; they evolved almost identical equations. Inspection of Van Slyke's scatter-diagram shows that the nine points from normal subjects are about 0·25 g. of protein per 100 ml. of serum above the correlation line. Later workers have used Van Slyke's equation for normal serum protein estimation (e.g. Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald‡), but Zozaya§ and Looney‖ found that the relationship was ill-defined. All workers have used the conventional nitrogen-protein factor of 6·25, for which there is little conclusive evidence.

The nature of the serum used and the methods of protein estimation do not then allow either Weech's or Van Slyke's equation to be used with confidence for normal human serum, and we have therefore made some preliminary comparisons of normal

human serum protein, estimated gravimetrically, with density, which gave the regression line, for eleven samples only:

$$\text{Protein} = 301{\cdot}1\ (\text{density} - 1{\cdot}0027),$$

with correlation coefficient of 0·986; protein is expressed as g./100 ml. of serum at 20°, and density is relative to water at 20° having density 1·00000.

This preliminary equation gives results higher than Van Slyke's by about 0·5 g. protein per 100 ml. in the normal range.

We have found in preliminary experiments that there is a closer relation between density and gravimetric protein than between density and micro-Kjeldahl nitrogen, and between gravimetric protein and density than between gravimetric protein and micro-Kjeldahl nitrogen.

In these experiments we have estimated density by the method of Jacobsen & Linderstrøm-Lang.¶ This is quick and reliable: measurements of density, correct to one part in ten thousand, may be obtained on a hundred samples of serum in about two hours. The copper sulphate method of Phillips *et al.*‡ is slower, less accurate and requires more serum, and we doubt if it gives a reliable estimate of density. First, the figures given for the density of $CuSO_4$ solutions made up from a saturated solution seem to us, from experimental measurements in the gradient, to be approximately 0·0005 high in the normal serum range. Secondly, with $CuSO_4$ densities corrected in this way, the $CuSO_4$ method gives results that are 0·0010 lower than those given by the gradient (n, 28; S.E. $\pm$0·0001). To check this, samples of plasma were diluted with water to float exactly in one or other of the $CuSO_4$ solutions. Direct comparison of plasma and its corresponding $CuSO_4$ solution in the gradient showed a mean difference of 0·0014 (n, 4; S.E. $\pm$0·0001), in fair agreement with the difference of 0·0010 given above.

In summary, a critical examination and preliminary experimental evidence show that the published equations for density-protein conversion are inadequate for normal human sera. The $CuSO_4$ method of estimating density has defects not shown by the gradient method.

* Moore, N. S. & Van Slyke, D. D. (1930). *J. clin. Invest.* **8**, 337.

† Weech, A. A., Reeves, E. B. & Goettsch, E. (1936). *J. biol. Chem.* **113**, 167.

‡ Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B. & Archibald, R. M. (1943). *Copper Sulphate Method for Measuring Specific Gravities of Whole Blood and Plasma.*

§ Zozaya, J. (1935). *J. biol. Chem.* **110**, 599.

‖ Looney, J. M. (1942). *J. lab. clin. Med.* **27**, 1463.

¶ Jacobsen, C. F. & Linderstrøm-Lang, K. (1940). *Acta physiol. scand.* **1**, 149.

Changes on Storage in the Biological Value of the Proteins of Dried Skim Milk.

By K. M. Henry and S. K. Kon

While a certain amount of information is available on changes in the biological value of the proteins of various food products brought about by heat treatment, little is known about the effects of storage, nothing, as far as we are aware, regarding dried milk.

Since 1941 we have examined on several occasions by the method of Mitchell* on groups of 12 rats the biological value of the proteins (nitrogen) of a sample of spray dried skim milk manufactured in 1939, and since stored at room temperature. The results are given below.

Test no.	Length of storage (months)	Biological value	True digestibility
1	19	88·5	89·8
2	40	81·3	87·6
3	43	80·3	96·9
4	49	75·0	88·1
5	54	71·1	88·1

Normally, dried skim milk would not be stored for such prolonged periods but it is generally accepted as a product of great stability and our observations may have a practical bearing. They are in agreement with similar findings regarding the effects of storage on the proteins of maize and of soya bean.† It should be noted that the true digestibility of the proteins of the milk remained unimpaired. Further work on the nature of the changes is in progress.

* Mitchell, H. H. (1924). *J. biol. Chem.* **58**, 873; Mitchell, H. H. & Carman, G. G. (1926). *J. biol. Chem.* **68**, 183.

† Mitchell, H. H. (1944). *Industr. Engng Chem.* (Anal. ed.), **16**, 696.

DEMONSTRATION

Densitometric Methods of Estimating Total Serum Protein. By B. B. Lloyd, H. M. Sinclair and G. R. Webster

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The **241st Meeting** *of the Society took the form of a Joint Discussion with The Nutrition Society on 'The Vitamin B Complex', and was held in the School of Hygiene and Tropical Medicine, Keppel Street, London, W.C.* **1**, *on Saturday,* 28 *April* 1945. *The following communications were given:*

Introductory Survey. By L. J. Harris and E. Kodicek

The attached table summarizes the extent of our present knowledge and the principal gaps in it. The following points among others are specially noted.

Name	Structural formula	Biochemical action	Needed by: Exp. animals	Needed by: Man	Deficiency disease in man	Approximate minimal human requirement	Estimation methods: Chem.	Estimation methods: Biol.	Estimation methods: Microbiol.
Aneurin (thiamin, vit. B_1)	√	Co-carboxylase	√	√	Beriberi	1 mg.	√	√	√
Nicotinamide	√	Co-dehydrogenase	√	√	Pellagra	12 mg.	√	√	√
Riboflavin	√	Co-dehydrogenase	√	√	'Cheilosis'	1·8 mg.	√	√	√
Pyridoxin (-al, -amine; vit. B_6)	√	? Transamination · Amino-acid decarboxylation	√	—	—	—	√	√	√
Pantothenic acid	√	—	√	—	—	—	—	√	√
p-Aminobenzoic acid	√	—	(√)	—	—	—	√	—	√
Inositol	√	—	(√)	—	—	—	√	—	√
Choline	√	Transmethylation	[√]	[√]	—	—	√	—	√
Biotin (vit. H)	√	—	√	√	—	—	—	√	√
'Folic acid'	—	—	√	—	—	—	—	√	√
Grass-juice factor	—	—	√	—	—	—	—	—	—

( )=? secondary accessory. [ ]=replaceable.

Vitamin B_1, as pyrophosphate ester (co-carboxylase), catalyzes the decarboxylation of pyruvic acid, but it can also be concerned directly or indirectly in numerous other complex and interrelated reactions, in which, however, it is possible that decarboxylation or carboxylation may enter at some stage as an essential feature. Clinically, crystalline vitamin B_1, if given in time and in sufficiently large doses, is successful in treatment of beriberi and conditioned deficiencies (e.g. polyneuritis associated with pregnancy, alcoholism, and gastro-intestinal obstruction). The vitamin (and others of B group) can

be synthesized by micro-flora in the alimentary canal in ruminants, in refected rats and sometimes to a variable extent in the human intestine. Several methods of estimation, biological and chemical, have been found to give concordant results. For assessment of nutritional status, measurement of excretion of B_1 after test dose, or tolerance curves of the rise in bisulphite-binding substances after loading with product of intermediate metabolism (lactic acid for rat, or glucose for man) are satisfactory methods.

B_2 complex. 'B_2' was first thought to be a single substance and defined as the heat-stable factor, present, for example, in yeast, and preventing (1) pellagra in man and blacktongue in dogs, (2) so-called 'pellagra-like dermatitis' in rats, and (3) needed for growth and maintenance of health in rats. Riboflavin (rat-growth factor) and vitamin B_6 ('rat pellagra' factor) were in turn differentiated from the true pellagra factor which prevents pellagra in humans, blacktongue in dogs and pellagrous conditions in monkeys and pigs. It was later identified with nicotinamide, and afterwards further differentiated from the so-called 'chicken pellagra' factor (pantothenic acid).

Nicotinamide is known to function as hydrogen transporter in dehydrogenase systems (as di- and tri-phosphopyridine nucleotides). For chemical and microbiological estimation, procedures must be used which include the biologically active chromogen (precursor) present in plant tissues. For assessing nutritional status nicotinamide may be given by mouth and the methylated product excreted in the urine determined either colorimetrically or fluorimetrically.

Riboflavin, in the form of flavo-proteins, functions in dehydrogenase systems. Clinical relations are still somewhat obscure, but deficiency is marked by cheilosis and corneal vascularization. In estimations by the microbiological technique attention is needed to interfering factors. For assessment of status a urinary excretion test is available.

Vitamin B_6 (pyridoxin) is concerned in protein metabolism (synthesis of fat from protein and tryptophane metabolism). *Pyridoxal* catalyses tyrosine decarboxylation, and, together with *pyridoxamine*, biological transaminations. 4-*Pyridoxic acid* lactone (excreted in human urine), in the presence of other accessory factors, is needed by chicks for preventing anaemia and promoting weight gains.

Pantothenic acid is a growth factor for numerous micro-organisms, and prevents 'chick pellagra', and loss of growth and greying of fur, and adrenal lesions in rats and mice.

p-Aminobenzoic acid and *inositol* may be 'secondary accessories' (i.e. not needed directly by mammals, but stimulating micro-organisms to synthesize other vitamins which are needed), although inositol appears to have lipotropic properties for rats.

Choline, concerned in transmethylations is replaceable by methionine and other substances.

Biotin (*vitamin H*) prevents seborrhoeic dermatitis in rats given raw egg-white (containing the anti-vitamin, avidin, which inactivates the vitamin), and it is also needed by man, yeast and bacteria.

Folic acid and other 'grass factors'. This heading comprises a group of apparently related substances, viz. folic acid, growth factors for *Lactobacillus casei*, and *Streptococcus lactis R*, vitamin M, vitamin B_c and perhaps xanthopterin (functioning as precursor).

Anti-anaemia factors include the extrinsic factor for pernicious anaemia, and factors preventing tropical macrocytic anaemias.

Some Recently Characterized Members of the B_2 Complex. By F. A. Robinson

The vitamin B_2 complex can be divided, somewhat arbitrarily perhaps, into three subgroups. The first comprises nicotinic acid and riboflavin which, together with aneurine, are associated with more or less clearly defined deficiency diseases. The second subgroup includes pyridoxine, pantothenic acid, biotin, folic acid, and *p*-aminobenzoic acid, none of which has yet been shown to be responsible for a specific disease. Pyridoxine was first

recognized as a factor capable of curing dermatitis in rats, and was subsequently shown to be a growth factor for micro-organisms. Pantothenic acid, biotin, and folic acid, on the other hand, were first identified as growth factors for micro-organisms, and later shown to be identical with dietary factors essential for animals. Pantothenic acid prevents dermatitis in chicks and biotin a dermatitis in rats caused by feeding egg-white. Folic acid cures the anaemia and leucopenia caused by administering sulphonamides to rats, and is probably identical with vitamin B_c, a factor necessary for the growth of chicks. It appears to be closely related to xanthopterin, a factor that cures anaemia in trout, and to vitamin M, a factor that cures anaemia and leucopenia in monkeys fed on a synthetic diet. *p*-Aminobenzoic acid is known to be a growth factor for micro-organisms and a factor that prevents the greying of hair in rats fed a synthetic diet; its biological importance was discovered as the result of its ability to counteract the antibacterial effect of sulphanilamide. Pyridoxine, pantothenic acid, biotin, and of course *p*-aminobenzoic acid have all been synthesized, but the structure of folic acid is not yet known with certainty. It is believed to be similar to that of xanthopterin, which has been synthesized. The function of these vitamins is not yet clear, but recently pyridoxal, a derivative of pyridoxine, was shown to be a constituent of tyrosine decarboxylase, and it has been suggested that folic acid may be present in the enzyme that elaborates thymine. With the possible exception of pyridoxine, the members of the second group are synthesized by the intestinal flora of experimental animals, and this may be the main source of supply of these factors. That these are mainly supplied in this way may in fact be a significant distinction between them and the members of the first subgroup, and may account for the fact that they are not associated with specific deficiency diseases. The third subgroup of the vitamin B_2 complex includes inositol, choline and possibly methionine. All three are lipotrophic factors, that is, they prevent the formation of fatty livers produced by feeding various supplements to experimental animals. Inositol is also a growth factor for micro-organisms, and its absence from the diet of rats causes loss of hair. The way in which these substances act is still obscure.

Parts Played by Members of the Vitamin B Complex in Enzyme Reactions. By J. H. QUASTEL

Aneurin. Aneurin-pyrophosphate is necessary for the oxidation and decarboxylation of pyruvic acid in animal tissues, in yeasts and in bacteria, and it is synthesized from aneurin by the living cell probably through the mediation of adenylpyrophosphate. Many biochemical changes demanding the participation of pyruvic acid, such as the synthesis of carbohydrate or of citric acid or of acetylcholine require the presence of aneurin-pyrophosphate. In fact, the central position which pyruvic acid occupies in many metabolic events makes the presence of aneurin a controlling factor in the kinetics of tissue metabolism. The mechanism by which aneurin pyrophosphate accomplishes its coenzyme functions is still unknown. It appears that the various specific proteins with which the vitamin is linked have activating actions on α ketonic acids, whereby oxidations, decarboxylations and synthetic transformations may be accomplished. The oxidation of acetic acid in bacteria appears also to be dependent on aneurin pyrophosphate. The presence of the magnesium ion is necessary for these changes and the potassium ion may also be an important accessory factor.

Transamination involving the participation of pyruvic acid or oxalacetic acid does not require the presence of aneurin-pyrophosphate.

Nicotinic acid. This substance was thrown into prominence by the discovery of Warburg and Christian that it is a constituent of a coenzyme of hexosemonophosphate oxidation. It appears to function in the cell as a coenzyme of oxidation in two forms, a

diphospho-nicotinamide-adenine-dinucleotide (cozymase) and a similar molecule containing three phosphoric acid groups instead of two. The nicotinamide moiety of the molecule undergoes reduction and oxidation by a variety of specific proteins (dehydrogenases) in presence of their substrates and the catalytic function of the coenzyme depends upon this phenomenon. Cozymase is the coenzyme of a large number of dehydrogenases with which it combines in a reversible way; this applies also to the triphospho analogue of cozymase. Cozymase, in the cell, acts as a carrier, transferring hydrogen from one set of substrates activated by their dehydrogenases to another set of molecules activated in a similar manner; it helps to control the speed and course of hydrogen transfer and in this way controls intracellular oxidations. It is presumably built from nicotinic acid supplied in the diet. Its content in the cell may also be influenced by the presence of free nicotinamide which inhibits specifically a tissue hydrolytic enzyme which breaks down cozymase.

Riboflavin. This forms a constituent of another coenzyme, *iso*alloxazine-adenine-dinucleotide, which in co-operation with various specific proteins is responsible for a variety of oxidation processes in the cell. The coenzyme is usually firmly bound to different proteins forming flavoproteins which bring about the oxidation of aldehydes, purines, amino-acids and the reduced forms of cozymase and its triphospho analogue. The seat of the catalytic power of the coenzyme lies in the *iso*alloxazine nucleus, which like that of nicotinamide, undergoes reduction and oxidation. The group of specific proteins with which the coenzyme is associated is in fact another class of dehydrogenases. The reduced form of flavoproteins is autoxidizable and unaffected by cyanide. It is, however, now held that in the living cell at the oxygen tensions normally obtaining in tissues a flavoprotein acts as a carrier between the reduced forms of the nicotinamide-adenine-dinucleotides and the cytochrome system.

Nicotinamide and *iso*alloxazine, in the form of their coenzymes and in co-operation with their specific dehydrogenases, help to control the interplay of intracellular oxidation systems whether the cell is existing under aerobic conditions or not.

Pyridoxin. The phosphorylated derivative of pyridoxal appears to act as a coenzyme of amino-acid decarboxylases.

Biotin and pantothenic acids. Little is known of the parts played by these substances in enzyme processes. They seem to be concerned in aspects of carbohydrate metabolism.

B Vitamins as Growth Factors for Micro-organisms and Related Growth-inhibitors. By B. C. J. G. KNIGHT

The study of the nutrient requirements of micro-organisms has shown that certain of the group B vitamins of higher animals are growth factors for micro-organisms, and conversely, certain growth factors discovered for micro-organisms function in animal nutrition. There appear to be fundamental metabolic reactions common to all cells which are carried out by similar systems of enzymes. Growth factors and vitamins in many cases are parts of these enzyme systems, which the cells must possess for normal metabolic functioning. But cells differ in the modes whereby these enzyme systems are acquired, namely by synthesis as part of their own metabolic activity or by partial synthesis and utilization of preformed structural units obtained from the nutrient environment. Nutritional requirements thus reflect the cell's ability to synthesize essential metabolites (parts of essential enzyme systems and structures, etc.) which are common to the widest variety of organisms. Comparative studies with micro-organisms can indicate possible routes of biosynthesis of essential metabolites. Growth inhibitors modelled on the structures of known essential metabolites can prove useful tools in the study of the biosynthesis of essential metabolites, and the interrelations of metabolic processes.

Fermentation and Human Nutrition. By B. S. PLATT and R. A. WEBB

The role of fermentation in human nutrition can be divided into two aspects, the fermentative changes occurring in foodstuffs before ingestion, and the changes occurring in unabsorbed residues of ingested food. For the most part they involve principally carbohydrates, although some important ones are known in which proteins are the principal substrate.

Fermented foods are found in various forms all over the world. A list showing the distribution of some representatives of each type of fermentation was given.

The fermentative changes can be considered to take place in three stages:

(1) The preparation of the raw material for fermentation, if it is not directly fermentable. This stage may lead to increase in B vitamins.*

(2) The proliferation of micro-organisms with concomitant synthesis of B_2 vitamins and proteins.

(3) The accumulation of end products of fermentation, alcohol, acids, and gas; leading to chemical and bacteriological stabilisation of nutrients in the foodstuff.

The stages of fermentation were illustrated by recent work of the authors on Kaffir beer (unpublished).

Fermentation in the intestines can be considered in terms of these same stages. There are differences, however, notably that in stage 1; enzyme action and absorption from the gut determine the nature and composition of the material available as a substrate for intestinal micro-organisms. The nature and composition of the residue plays a large part in determining the microbiological flora of the gut, as well as the products of the action of a given flora.

It has been shown that intestinal micro-organisms can, *in vitro*, synthesize considerable amounts of several B vitamins.† There is evidence that synthesis of B_1‡, riboflavin§ and nicotinic acid‖ occurs in the gut of man. The question of the availability of these synthesized vitamins to the individual is not solved yet. Doubt has been cast on the availability of B_1 from this source.¶ The question is complicated by the fact that existing physiological studies do not account for more than a small proportion of the various vitamins ingested.

The possible extent of bacterial action in the intestines can be gauged from the weight of bacterial bodies excreted daily in faeces, as well as from the determination of products of their action absorbed by the human organism. Some figures obtained by the authors of the alcohol content in the circulating blood of human subjects following a carbohydrate meal were given, showing a significant increase 6–13 hr. after the meal. It was suggested that this might be due to bacterial fermentation in the gut.

The significance of fermented foods in practical nutrition was demonstrated by a comparison of a simple African maize diet with the same diet in which a portion of the maize was replaced by an equivalent of maize beer. This substitution leads to an increase, notably in riboflavin and nicotinic acid from an insufficient amount to a more than adequate amount by present-day standards. As a contrast, a table of the incidence of symptoms of nutritional deficiencies in a community (Barbados) which does not have a fermented food comparable with the Kaffir beer of their forebears was shown (from a recent survey by B. S. Platt). It was shown how this state could be ameliorated by increasing the extraction rate of the flour and the addition of food yeast.

Later in the meeting a number of colour slides showing the clinical picture of the types of nutritional deficiency symptoms encountered in Barbados and Newfoundland were shown by B. S. Platt.

* Burkholder, P. R. (1942). *Science*, **97**, 562.
† Burkholder, P. R. *et al.* (1943). *Food Res.* **8**, 314.
‡ Najjar, V. A. & Holt, L. T. (1943). *J. Amer. Med. Ass.* **123**, 683.
§ Najjar, V. A. *et al.* (1944). *J. Amer. Med. Ass.* **126**, 357.
‖ Ellinger, P. & Benesch, R. (1945). *Lancet*, **248**, 432.
¶ Alexander, B. & Landwehr, G. (1945). *Science*, **101**, 229.

B_2 Vitamins and Anaemia. By L. WILLS

The paper was chiefly devoted to a consideration of the relation of the deficiency giving rise to nutritional macrocytic anaemia to some at present unidentified components of the vitamin B_2 complex, and to Castle's extrinsic factor. It was pointed out that the blood pictures in Addisonian pernicious anaemia and nutritional macrocytic anaemia are identical, and that it is generally thought that both anaemias are due to a deficiency in the liver principle resulting from a lack of the intrinsic and extrinsic factors respectively. Uncomplicated cases of nutritional macrocytic anaemia, however, do not respond to highly purified liver extracts known to be curative in true pernicious anaemia,* though they respond well to crude liver extracts given parenterally and to autolyzed yeast extracts (marmite) by mouth.

Experimental work done on monkeys confirmed the clinical findings.† A nutritional macrocytic anaemia was produced by feeding a deficient diet and this anaemia failed to respond to purified liver extracts though crude liver and autolyzed yeast extracts given either parenterally or by mouth were highly active curatively. An attempt was made to identify the nature of the missing factor in the diet by therapeutic trials. Vitamin B_1 was neither preventive nor curative. Fractions containing different components of the vitamin B_2 complex were prepared by Wing-Commander Macrae from liver extracts kindly supplied by the Glaxo Laboratories. Nicotinic acid, riboflavin pyridoxine, pantothenic acid and *para*-amino benzoic acid were excluded as the active factor both by direct trial of the pure substance in some cases and by giving fractions free from these components. The outbreak of war interrupted the work, but incomplete experiments suggested that a mercuric acetate precipitate free from the above-mentioned fractions contained the curative factor.

Handler & Featherstone‡ produced a nutritional macrocytic anaemia by feeding dogs on different nicotinic acid-deficient diets. They argue from their results that a nicotinic acid deficiency acts by interfering with the cellular metabolism of the erythroblasts through a resulting lack of cozymase. It was suggested that a similar interference in cellular metabolism due to a lack of some component of a cellular enzyme system may be the cause of the macrocytic anaemia in man and monkeys, but that the factor is different and at present unidentified. Further, the factor is not identical with Castle's extrinsic factor§ as it is active when given parenterally and purified liver extracts active in pernicious anaemia are inactive in the nutritional macrocytic anaemia.

* Wills, L. & Evans, B. D. F. (1938). *Lancet*, **2**, 416. Napier, L. E. (1939). *Ind. Med. Gaz.* **74**, 1.

† Wills, L., Clutterbuck, P. W. & Evans, B. D. F. (1937). *Biochem. J.* **31**, 2136.

‡ Handler, P. & Featherstone, W. P. (1943). *J. biol. Chem.* **151**, 394.

§ Dr E. Kodicek in the discussion said that Castle had recently agreed that the factor whose lack produces nutritional macrocytic anaemia is not identical with his extrinsic factor.

The **242nd Meeting** *of the Biochemical Society was held in the Institute of Physiology, University of Glasgow, on Saturday,* 9 *June* 1945, *at* 11.30 *a.m., when the following communications and demonstration were given:*

COMMUNICATIONS

Plasma Bilirubin in the Horse. By W. N. M. RAMSAY

Plasma bilirubin determinations (method of Haslewood & King*) have been made on 77 apparently normal blood specimens from 51 horses. The mean of the series was 1·57 mg./100 ml., and the S.D. 0·86 mg./100 ml. The lowest value was 0·48 mg./100 ml., and two-

thirds of the values were above 1 mg./100 ml. These rather large concentrations of bilirubin have been shown to account for the high colour of horse plasma compared with that of other species. Bilirubin in horse plasma shows a broad absorption band with a maximum about 470 mμ.

Fasting for 48 hr. causes a striking increase in plasma bilirubin in the horse. In one case, for example, the concentration remained constant for 6 days at 0·76–0·83 mg./100 ml. 48 hr. fasting increased the level to 1·86 mg./100 ml. Six other experiments gave similar increases, the greatest being from 1·96–5·1 mg./100 ml. When the animals are fed, the bilirubin concentration falls again.

* Haslewood, G. A. D. & King, E. J. (1937). *Biochem. J.* **31**, 920.

The Effects of Methylation on the Activity of Nitrogenous Carcinogens. 1. *p*-Aminoazobenzene in the Rat. By A. H. M. Kirby

In an attempt to explain the carcinogenic action of azo-dyes on the liver of rats, Kensler *et al.** compared the degree of carcinogenicity of some of these dyes with the degree of inhibition of a DPN system by the partially oxidized form of the *p*-diamines obtained by reductive fission. Their table has been severely criticized by Miller & Baumann† who have also shown that *N*-methyl-*p*-aminoazobenzene is as carcinogenic as butter-yellow though its fission-produced diamine is much less inhibitory.

Since the unmethylated azo-dye, 4′-amino-2:3′-azotoluene (*o*AAT) is a liver carcinogen for rats, *N*-methylation does not seem to be essential; further, as the fission-product of *p*-aminoazobenzene (AAB) is also an enzyme inhibitor, it seemed possible that the latter dye is a carcinogen too. Sasaki & Yoshida‡ fed AAB to rats for 14 months without eliciting tumours. However, when this dye was fed to 16 Wistar rats at a level of 200–300 mg./100 g. diet,§ one rat died after 13 months with small, localized hepatomas and no cirrhosis. Two rats dying after 17 months each had massive tumours in one ventral lobe and small tumours in the other lobes of the liver. In one case the liver plus tumours weighed 47·8 g. while the spleen weighed 5·75 g. These two livers presented the following conditions: perilobular cirrhosis, cholangioma, malignant hepatoma associated with angioma and haemorrhage into the peritoneal cavity. Secondary hepatoma in the mesentery was found in one rat; primary lung adenoma in the other. Other rats dying later showed only small hepatomatous nodules; cirrhosis was not a constant finding.

Thus it appears that methylation of the primary amine, AAB, enhances but does not initiate carcinogenic activity. This is in accordance with the 'split-product' theory of Kensler *et al.* However, the findings of Miller & Baumann that the *p*′-methyl derivative of butter-yellow is much less active than the parent dye, while the *m*′-methyl derivative is even more powerful than butter-yellow itself, although all three would yield the same diamine, shows that the 'Split-product' theory will not cover all the facts. Moreover, Miller *et al.*‖ present evidence that demethylation may precede fission *in vivo*, in which event the highly inhibitory *N*:*N*-dimethyl *p*-phenylenediamine would never be formed.

* Kensler, C. J., Dexter, S. O. & Rhoads, C. P. (1942). *Cancer Res.* **2**, 1.
† Miller, J. A. & Baumann, C. A. (1945). *Cancer Res.* **5**, 227.
‡ Sasaki, T. & Yoshida, T. (1935). *Virchows Arch.* **295**, 175.
§ Kirby, A. H. M. (1944). *Nature, Lond.*, **154**, 668.
‖ Miller, J. A., Miller, E. C. & Baumann, C. A. (1945). *Cancer Res.* **5**, 162.

The Effects of Methylation on the Activity of Nitrogenous Carcinogens. 2. Azo-dyes and *N*-methyldibenzcarbazole in Mice. By A. H. M. Kirby

Liver tumours were not induced in stock mice of mixed colours which were injected once a fortnight with 0·25 ml. of a 3 % solution of *p*-aminoazobenzene in arachis oil; one male mouse survived 626 days and received a total of 192·5 mg. of the dye, but showed no abnormality of the liver. Acute to chronic toxic nephritis was often found in these mice.

An isomer of *o*AAT, 2′-amino-4:5′-azotoluene was reported by Sasaki & Yoshida to be non-carcinogenic when fed to rats. This also appears to be true for stock mice; the dye was injected subcutaneously in olive oil once a fortnight. Mice survived up to 472 days, by which time 67·5 mg. had been administered. Local or massive necrosis of the liver was frequent, but there was no evidence of regeneration as is seen with *o*AAT and butter-yellow; toxic effects on the kidney were also absent. This dye (and also AAB) might be carcinogenic for mice if the treatment were very prolonged.

Whereas butter-yellow is much more carcinogenic than is *o*AAT when fed to rats, the reverse appears to be true for mice. Law* showed this for *dba* and C57 Black strains of mice receiving these dyes by subcutaneous injection, and Andervont & Edwards† confirmed it for mice of A, C, C3H and C57 Black strains. This work has now been extended with similar results to stock mice of mixed origin and to mice of the CBA strain which are liable to spontaneous hepatoma at an advanced age. If the 'split-product' theory of Kensler *et al.* is true for rats, it cannot be true for mice in which the relative carcinogenicities of these two azo dyes is reversed. Sarcomas at the site of injection were reported by Law and by Andervont & Edwards for both dyes and for *o*AAT by Turner & Mulliken‡ in strain C mice; sarcomas have also now been found in stock mice and in C57 Black female mice with either dye, but none in either sex of C*ba* strain mice.

In the dibenzcarbazoles, nitrogen is part of the ring structure. Although 3:4:5:6-dibenzcarbazole (DBC) is highly carcinogenic when painted on the skin of mice§ or injected subcutaneously,‖ there can be no question of the formation of a fission product of a diamine type as in the azo-dyes. Since methylation of AAB to butter-yellow (*N*:*N*) or to *o*AAT (*C*:*C*′) increases the carcinogenicity, the effect of *N*-methylating DBC is of interest. *N*-methyl DBC (MDBC) was synthesized by the general method of Stevens & Tucker,¶ and has m.p. 148° C. It was administered to stock mice either by painting twice weekly in acetone solution (0·2%) or by a single subcutaneous injection of 0·4 mg. in arachis oil. Controls were treated in parallel with the same amounts of DBC. Papillomas appeared on the skin in 3–4 months with DBC; the latent period was extended to 7½–8 months with MDBC. Sarcomas appeared after 3½ months in 12/12 mice receiving DBC, and after 5 and 10 months respectively in 2/12 receiving MDBC, 10 mice still surviving. Whereas DBC evoked regeneration, hepatomas and cholangiomas, MDBC only caused fatty degeneration and varying degrees of necrosis of the liver.

It is proposed to extend these experiments to cover 2-aminofluorene;** *N*-monomethyl-2-aminofluorene has been synthesized and has m.p. 181° C.

* Law, L. W. (1941). *Cancer Res.* **1**, 397.
† Andervont, H. B. & Edwards, J. E. (1943). *J. Nat. Cancer Inst.* **3**, 349.
‡ Turner, J. C. & Mulliken, B. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 317.
§ Boyland, E. & Brues, A. M. (1937). *Proc. Roy. Soc.* B, **122**, 429.
‖ Strong, L. C., Smith, G. M. & Gardner, W. U. (1938). *Yale J. biol. Med.* **10**, 335.
¶ Stevens, T. S. & Tucker, S. H. (1923). *Trans. Chem. Soc.* **123**, 2140.
** Bielschowsky, F. (1943). *Brit. J. exp. Path.* **25**, 1.

Capillary Resistance and Social Level in Glasgow. By H. SCARBOROUGH

Ethyl Gallate and Ascorbic Acid as Antioxidants in Dried Whole Milk. By J. D. FINDLAY and J. C. D. WHITE

Milk powder was made from one batch of full-cream milk with and without the addition of very small amounts of ethyl gallate and ascorbic acid. Both these compounds lengthened the storage life of the powder very materially, ethyl gallate causing a greater extension of storage life than ascorbic acid. During storage the concentration of ethyl gallate in the powder remained unchanged, but the ascorbic acid gradually decreased.

The Value of Incubations *in vitro* applied to the Study of Ruminant Digestion. By MARY L. MCNAUGHT and J. A. B. SMITH

Pearson & Smith* and Baker & Smith† have shown that during the incubation of rumen contents, some N.P.N. is converted to protein. It was important to know whether the results of *in vitro* incubations of this type applied equally to rumen contents in the intact rumen. A study has therefore been made of the formation of CO_2, CH_4, lactic acid and lower fatty acids during typical *in vitro* incubations. The formation of these substances by the rumen contents in the laboratory has been found to be similar to that reported by others for the intact rumen. The fact that the formation of these substances in the laboratory is always accompanied by the conversion of N.P.N. to protein, supports the view that the conversion of N.P.N. to protein also occurs in the intact rumen.

* Pearson, R. M. & Smith, J. A. B. (1943). *Biochem. J.* **37**, 153.
† Baker, F. & Smith, J. A. B. (1944). *Biochem. J.* **38**, 496.

The Effect of Thyroxine Injections on the Nitrogen and Calcium Metabolism of the Lactating Cow. By E. C. OWEN

Thyroxine is known to cause an increase in the yield of milk and milk fat when administered to cows in declining lactation. Proteins iodinated under controlled conditions are known to affect metabolism in a very similar way to thyroxine. Since the proteins might be used in practice to increase the production of milk and milk fat, it became important to determine what effect thyroxine administration has upon the nitrogen and calcium metabolism of lactating cows. Of six cows kept in a special metabolism house, four were injected with thyroxine daily for one month, and two on the same diet were kept as controls. With the injected cows substantial increases in the yield of milk and fat were accompanied by negative nitrogen balances. The nitrogen balances became markedly positive when the injections were stopped.

Null-point Detector for Photoelectric Absorptiometers. By G. H. BELL and W. MCFARLANE (introduced by G. M. WISHART)

The balanced version of the photoelectric colorimeter (or absorptiometer) described by Bell & Guthmann* was neither portable nor compact since it used a moving-coil reflecting galvanometer. By replacing the galvanometer by a null detector consisting of a pentode amplifying valve, 6C6, directly coupled by resistance to an electron beam tuning indicator, EM1, it is possible to construct a self-contained portable outfit. A variable resistor connected in the cathode lead of the pentode permits of a fine adjustment of its anode current which in turn controls the voltage on the grid of the EM1 and the width of the illuminated sector. With the input short-circuited the illuminated sector is brought to half the maximum width. When the shorting switch is open the application of any out-of-balance potential difference from the photocells to the grid of the pentode alters the width of the sector and thus the null point is easily found. The sensitivity of the EM1 can be controlled by the insertion of a variable cathode resistor.

* Bell, G. H. & Guthmann, E. (1943). *J. sci. Instrum.* **20**, 145.

The Effect of Purines on Polycyclic Aromatic Hydrocarbons. By H. WEIL-MALHERBE

Some of the biologically most active substances are insoluble in water. Unless it is assumed that they display their activity in a lipid phase, there must be a mechanism whereby hydrophilic properties are conferred on them. In this connexion the remarkable solvent effect of caffeine on 3:4-benzpyrene, discovered by Brock, Druckrey & Hamperl* is of particular interest. This effect has been studied quantitatively; at the same time the investigation was extended to a wide range of other purines and hydrocarbons.

The findings may be summarized as follows: the solvent effect of purines in aqueous solution on, for example, 3:4-benzpyrene increases (*a*) with the number of substitutions by oxygen of the positions 2, 6 and 8 of the purine ring, (*b*) with the number of *N*-methyl groups. Hypoxanthine, with the weakest solvent power, stands at one end of the scale, tetramethyl-uric acid, with the strongest one, at the other. Not only the number, but also the position of the substitution is of importance; thus, 3:7-dimethylpurines are superior to 1:3-dimethylpurines. Compounds with electronegative substituents, such as methoxy-, ethoxy- or Cl-derivatives, are intermediate between the corresponding compounds substituted by H- and those substituted by O-. The nucleosides adenosine and guanosine also show a solvent effect.

The solvent power of all those purines which have acidic properties is greatly reduced in alkaline solution, no matter which group is the seat of the acidic function. The solvent power of caffeine and tetramethyluric acid which have no acidic properties is unaffected by alkali.

Small modifications of the purine-ring structure, such as loss of the 8–9 double bond or replacement of trivalent N in position 9 by pentavalent N, results in great reduction or complete loss of solvent power.

The solvent power of any particular purine varies with its concentration. It was found that the molar concentration of benzpyrene in solution is roughly proportional to the square of the purine concentration, at least in the higher concentration ranges. The best fitting curves have exponents of 1·7–1·9. At low purine concentrations the character of the curves is changed.

A second effect of purines on polycyclic aromatic hydrocarbons which was found in these studies is a reversible quenching of fluorescence. Three groups can be distinguished: (1) purine bases, such as hypoxanthine, xanthine, dimethylxanthines, caffeine, etc., quench only in acid solution, or as acid salts, i.e. in ionic form. The quenching is completely reversible by neutralization. (2) N-methylated uric acids quench in absence of acid. Addition of alkali does not reverse the quenching. It is, however, reversible by separation of hydrocarbon and purine. (3) Caffeine derivatives with electronegative substituents in position 8, such as methoxy-, ethoxy- or chlorocaffeine, quench in absence of acid, but to a lesser degree than the substances of group 2. Addition of acid brings the quenching to the level of group 1+acid or of group 2.

The quenching effects are not due to any change in the dispersion or solution of hydrocarbon or to an absorption of either primary or secondary radiation.

* Brock, N., Druckrey, H. & Hamperl, H. (1938). *Arch. Exp. Path. & Pharmak.* **189**, 709.

Bone Strength and Ash Content of Femora of Rats on Rachitogenic Diet. By G. H. BELL and J. W. CHAMBERS

In previous experiments on rats no alterations were found in bone quality (as determined by mechanical tests) on diets with a wide variation in Ca content, nor did alterations occur following injection of hormones. The present investigation concerns the effects of rachito-

genic diet (Steenbock no. 2965) fed, with and without vitamin D supplements, to groups of litter mates for 4–5 weeks; in one experiment a further group of litter mates was fed on a full diet. The femora were measured, weighed and the breaking stress was determined; fragments of the shaft were analyzed for ash, Ca and PO_4. The averages for weight of femur, length of femur and percentage ash in femur were less in rachitic rats than in non-rachitic. The average values for percentage Ca in the ash and the Ca/P ratio were the same in all groups. Breaking stress on bending and breaking stress on twisting were less for the rachitic rats than for the non-rachitic. The value for breaking stress on bending was greater in rats on the full diet than in the non-rachitic group, but the breaking stress on twisting was the same in these two groups. The correlation between the values of percentage ash and breaking stress on bending over the whole experiment was 0·52 (S.E. 0·15). This indicates that the quality of the bone material is fairly closely related to the percentage ash in the bone.

The Investigation of Bone Structure by X-ray Diffraction. By I. M. DAWSON (introduced by J. W. CHAMBERS)

X-rays are diffracted by an ordered array of atoms, so that if in bone there is a regular molecular arrangement an X-ray diffraction photograph characteristic of that arrangement will be obtained. Specimens of normal and rachitic rat bone were examined and both gave the diffraction pattern characteristic of hydroxylapatite, $Ca_3(PO_4)_2\ xH_2O$, the inorganic constituent of bone. On removal of calcium salts, the bone protein which remained gave a pattern similar to that obtained from collagen. The hydroxylapatite crystallites and the amino-acid chains lie along the bone axis both in the normal and rachitic bones, the degree of orientation being the same in both cases. From the investigation it can be concluded that the chemical constitution and the orientation of the principal constituents of bone remain unaltered in rickets and that any change in bone strength is due to the change in the proportion of these constituents.

Polypeptidaemia. By J. N. DAVIDSON and W. C. WILSON

Whole blood contains nitrogenous material not precipitable by trichloroacetic acid or by sulphosalicyclic acid but precipitable by phosphotungstic acid, tungstic acid or alcohol. This material is usually referred to as 'polypeptide' or as 'proteose', although its exact composition is unknown and may be variable. It is present in normal fasting whole blood in a concentration of about 4 mg. N/100 ml. in man,* the dog† and the rat‡ and about 12 mg. N/100 ml. in the fowl.§ It has been reported in much higher concentrations in the blood of tumour-bearing rats‡ and is present in a concentration of about 18 mg. N/100 ml. in the blood of fowls bearing the Rous sarcoma.§ In a series of estimations by Burstein's method† on the whole blood and plasma of human patients with carcinoma or sarcoma it was found that raised values for 'polypeptide' N concentration were found in some cases only. High values were frequently found in association with acute infections especially where blood culture was positive, but the degrees of polypeptidaemia bore no relationship to the degree of leucocytosis. Very high values for 'polypeptide' N in whole blood, but not in plasma, were found in cases of leukaemia, reaching as high as 70 mg./100 ml. in the myelogenous type. As the white cell count fell in response to radiotherapy, the 'polypeptide' N concentration fell correspondingly.

* Arduino, J. C. (1941). *Rev. Fac. Cienc. quím. La Plata*, **16**, 191.

† Burstein, M. (1937). *J. Physiol. Path. gen.* **35**, 71.

‡ Winzler, R. J. & Burk, D. (1943). *Cancer Res.* **3**, 135.
Burk, D., Winzler, R. J. & Hesselbach, M. (1944). *J. Nat. Can. Inst.* **4**, 417.

§ Davidson, J. N. & Waymouth, C. (1944). *Brit. J. Exp. Path.* **25**, 164.

Effect of Young's Anterior Pituitary Extract on the Carbohydrate Metabolism of the Human Subject. By A. SLESSOR (introduced by N. MORRIS)

The results in four 'normal' subjects are presented each of which received 100–200 c.c. of Young's crude anterior pituitary extract over a period of 8–14 days. When the larger doses were being given the fasting blood-sugar level was raised, but there was no significant change in the intravenous glucose tolerance nor in the insulin sensitivity.

A single injection of 10 c.c. of the extract enhanced the hyperglycaemic action of subcutaneously administered adrenaline, but when the adrenaline was given by slow intravenous infusion, no enhancement was noted. In a case of pituitary cachexia, treated with anterior pituitary extract, the insulin sensitivity was diminished while the intravenous glucose tolerance appeared to increase.

Osteoporosis in Old Age. By C. M. KESSON and N. MORRIS

The criteria of osteoporosis are discussed and a brief reference is made to symptomatology. Little change has been noted in the blood chemistry. Metabolic investigations indicate an adequate retention of calcium and phosphorus. In spite of a high calcium intake over periods exceeding six months no significant change was noted in the X-ray pictures. Some other possible causes of osteoporosis are briefly discussed.

The Estimation of Serum Proteins. By H. HOCH and J. R. MARRACK

In the Kjeldahl method of estimating nitrogen in serum, we have used 0·2 ml. of serum, 0·17 ml. of 30% solution of copper sulphate, 1 ml. of sulphuric acid. After 10 min. digestion, add about 1 g. of sodium sulphate and, after cooling, 0·1 ml. of selenium dioxide solution (0·124 g. in 25 ml.) or an equivalent amount of sodium selenate. If the digestion was continued for 50 min. when selenate was used or for 3½ hr. when selenium dioxide was used, the results differed by about 1% from those obtained by prolonged digestion.

The specific gravities estimated by the copper sulphate method are not the true specific gravities, as estimated by weighing in a pycnometer. The results obtained with this method for 115 sera of normal blood donors, healthy pregnant women, women after delivery, returned prisoners of war suffering from semi-starvation and patients with various diseases fit the equation (77% within ±3%, 98% within ±5%)

$$P = 364\,(S - 1{\cdot}006),$$

where P is the concentration of protein in grams per 100 ml., and S is the specific gravity found.

Specific gravities found by a method resembling that of Jacobsen & Linderström-Lang,* but using chlorobenzene in place of bromobenzene, gives specific gravities that nearly agree with those found by direct weighing. The relation between protein concentration and specific gravity found by weighing is given by the equation

$$P = 366\,(S - 1{\cdot}007).$$

When bromobenzene is used the specific gravities found are slightly higher; this may be due to the solution of the heavy bromobenzene in the serum.

* Jacobsen, C. F. & Linderström-Lang, K. (1940–1). *Acta Physiol. Scand.* **1**, 149.

DEMONSTRATION

X-ray Diffraction Photographs of Bone Structure. By I. M. DAWSON (introduced by J. W. CHAMBERS)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **243rd Meeting** *of the Biochemical Society was held in the School of Medicine, Thoresby Place, Leeds, 2, on Friday, 20 July* 1945 *at* 2.30 *p.m., when the following communications were given:*

COMMUNICATIONS

The Effect of Various Solvents on the Rate of Elimination and Carcinogenic Activity of 3:4-benzypyrene. By F. DICKENS and H. WEIL-MALHERBE

Several authors have shown that, when used as a solvent for the subcutaneous injection of carcinogens, fat from the same species of animal may inhibit carcinogenesis.* Peacock & Beck (1938) attributed the loss of carcinogenic activity to increased rate of elimination. We have previously shown that lipids from other species may have the same anticarcinogenic effect; an extract of ox brain containing phospholipins and some cholesterol inhibited carcinogenesis in mice,† and purified lecithin and cephalin were also inhibitory.‡ We have now studied this more fully, and have determined the rate of elimination of 3:4-benzpyrene in various solvents.

A sample of mouse fat, partially purified so as to contain only 1·5 % of phospholipins, which previously inhibited carcinogenesis.† was tested again after keeping it in the laboratory for three years; it had then lost its anticarcinogenic action and its effect was similar to that of synthetic tricaprylin. This suggested that a labile component of the natural fat was involved, and the behaviour of unsaturated fats and phosphatides was therefore studied. A fresh sample of mouse fat, containing only 0·05 % of phospholipins, was prepared from dissected adipose tissue, and this did not diminish carcinogenesis. The anticarcinogenic effect of the original mouse fat was found to be duplicated by a dilute solution of phosphatides, containing 1·5 % lecithin and 1·5 % cephalin in tricaprylin. On the other hand, a 3 % solution of cholesterol in tricaprylin strongly enhanced the carcinogenic activity of benzpyrene.

This evidence showed that probably the greater part of the anticarcinogenic action of mouse fat is due to its content of phospholipins. That unsaturation was not responsible was shown by experiments with hydrogenated fats; also cod-liver oil, which is rich in polyethenoid fatty acids, was not anticarcinogenic.

The rate of elimination of benzpyrene from mice was measured by its chromatographic separation and fluorimetric estimation.§ By comparison with pure tricaprylin, elimination was significantly accelerated by the presence of dissolved cholesterol and delayed by the phosphatides. The high tumour incidence with the former and the low incidence with the latter are opposed to the view that slow elimination favours carcinogenesis.

It is presumed that this effect is primarily connected with the rate of oxidative metabolism of the carcinogen, which is depressed by phosphatides, probably through their antioxidant properties, and accelerated by cod-liver oil and cholesterol in varying degrees. Up to a certain limit, the rapid metabolism of the hydrocarbon favours carcinogenesis, while depression of carcinogen metabolism is anticarcinogenic. This would accord with the hypothesis that the hydrocarbon is not itself the true carcinogen, but that the active formation of an oxidized metabolite determines the activity. In this case the metabolite of the hydrocarbon must be considered as the true carcinogen.

* Watson, A. F. (1935). *Amer. J. Cancer*, **25**, 753; Peacock, P. R. (1935). *Proc. Leeuwenhoek Vereeniging, Amsterdam*; Peacock, P. R. & Beck, S. (1938). *Brit. J. exp. Path.* **19**, 315; Morton, J. J. & Mider, G. B. (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 357.

† Dickens, F. & Weil-Malherbe, H. (1942). *Cancer Res.* **2**, 560.

‡ Weil-Malherbe, H. & Dickens, F. (1944). *Cancer Res.* **4**, 425.

§ Weil-Malherbe, H. (1944). *Biochem. J.* **38**, 135.

The Metabolism of Sulphanilamide, Metanilamide and Orthanilamide. By R. T. WILLIAMS

These three sulphonamides undergo oxidation and acetylation *in vivo*. The extent of these processes in the rabbit is dependent on the position of the $—NH_2$ in relation to the $—SO_2NH_2$. Oxidation is greatest with orthanilamide and least with sulphanilamide, whereas for acetylation the reverse is true. The oxidation product in each case is an *o*-aminophenolsulphonamide excreted combined as an ethereal sulphate and glucuronide.

The aminophenolsulphonamides were isolated crystalline. Thus sulphanilamide yielded 3-hydroxysulphanilamide (3-hydroxy-4-aminobenzenesulphonamide) identical with an authentic sample; metanilamide yielded 4-hydroxy-3-aminobenzenesulphonamide identical with an authentic specimen; orthanilamide gave what is probably 3-hydroxy-2-aminobenzenesulphonamide, since a catechol compound was obtained on diazotizing and boiling the oxidation product.

Crystalline acetyl derivatives were isolated in each case. From orthanilamide urine, 3-methyl-[benzo-1:2:4-thiodiazine]-*l*-dioxide was also isolated, but this compound is probably an artefact formed by the dehydration of acetylorthanilamide.

The approximate distribution of metabolites in urine (rabbit) is as follows (doses 0·1–0·5 g./kg.):

	Orthanilamide % of dose	Metanilamide % of dose	Sulphanilamide % of dose
Oxidized and conjugated as ethereal sulphate	30–45	20–23	7– 9
Acetylated	*ca.* 10–20	30–50	40–80
Free diazotizable $—NH_2$*	40–80	30–50	10–50
Diazotizable $—NH_2$* after hydrolysis	35–75	70–80	70–90
Oxidized and conjugated as glucuronide	Large amount	Appreciable amount	Very small

* And coupling with naphthylethylenediamine.

3-Hydroxysulphanilamide has also been detected spectroscopically, in human sulphanilamide urine.

No evidence was obtained for the *in vivo* formation of *p*-hydroxylaminobenzenesulphonamide from sulphanilamide. Various fractions from the urine of rabbits, heavily dosed with sulphanilamide, were examined spectroscopically for azoxybenzene-4:4′-disulphonamide into which the hydroxylamino compound is rapidly converted in neutral or alkaline solution. The azoxy compound shows a large absorption band with a maximum at 333 mμ. No trace of this compound was found.

The glucuronides of the *o*-aminophenolsulphonamides are highly stable compounds and resist complete hydrolysis even when boiled for 4 hr. with 5N-HCl. Hence their estimation by the naphthoresorcinol/HCl method is not satisfactory.

The Estimation of Histidine and its Excretion in Normal Pregnancy. By F. W. CHATTAWAY

The accurate colorimetric estimation of urinary histidine is rendered difficult by the presence of urates and phosphates and by the pigmentation of the urine. It has been shown that strict control of pH eliminates some of the difficulties previously ascribed to these sources and further that two distinct pigments can be obtained, as the colour is developed at a pH value greater or less than 5. The existence of these substances can be demonstrated by titration curves of pigments prepared at varying pH values.

The excretion of histidine in the early morning sample of urine has been examined at weekly intervals during the course of a normal pregnancy. The excretion was higher at all times than in the non-pregnant condition and showed a peak during the fifth month. No general conclusions can be drawn from an examination of one case but the results are discussed in relation to previously recorded values obtained from examination of only a few samples per patient.

Nutritional Requirements of *L. casei* E. The Separation of Necessary Growth Factors. By F. W. Chattaway, Doris E. Dolby, Frank C. Happold, Mary McMillan and J. W. Waters

The separation of growth factors for *L. casei* E and for *C. diphtheriae* from the amyl alcohol insoluble fractions obtained when extracts of liver are made at pH 3 has been previously reported upon.*

Three separate factors have been clearly identified, none of which could be replaced by Folic acid (kindly supplied by Prof. R. J. Williams).† The separation of these factors from folic acid has been effected and synergistic action has been shown to occur between various pairs of these fractions.

Some of the chemical properties of these various factors have been established.

* Chattaway, Happold & Sandford (1944). *Biochem. J.* **38**, 111; Chattaway, Dolby & Happold (1944). *Biochem. J.* **38**, *Proc.* xviii; Dolby, Happold and Sandford (1944). *Nature, Lond.*, **153**, 619.

† Mitchell, Snell & Williams, R. J. (1944). *J. Amer. chem. Soc.* **66**, 267.

An Assessment of the Total Dietaries of Elementary School Children. Winter—Summer 1943–4. By F. W. Chattaway, Frank C. Happold and A. Margaret Happold

Enzymic and Antigenic Responses of *Esch. Coli* to Growth in Glucose-containing Media. By Frank C. Happold and J. W. Waters

It has been shown that modification of the conditions under which certain organisms grow modifies also the antigenic structure of the organism. Thus growth of *Salmonella aertrycke* in the presence of glucose inhibits the production of the '*X*' antigen of Topley & Ayrton (Happold*). Similar modification of a medium inhibits the production of the tryptophanase complex by *Esch. coli* though this adaptive enzyme system reappears when the level of stored polysaccharide in the cell drops below a certain concentration (Dawson & Happold†).

Is there a relationship between these phenomena? Is this stored polysaccharide to be regarded as a protective capsular layer, a part of the specific group of an antigen or antigens, which may even be present in the cell which has developed in non-glucose-containing media, but which, when developed in glucose-rich media, masks the presence of the *X* antigens (which incidentally reappear when the glucose in the medium has been utilized) and masks also a part of the enzyme complex of the cell? Our working hypothesis was that the above might happen but that the inhibition of the tryptophanase system was more likely to be due to competition for co-enzyme factors common to carbohydrate dismutation and to the oxidation of tryptophan to indole. Results show that unlike cultures of the salmonellas, cultures of *Esch. coli* grown in the presence of glucose reveal no antigenic differences to cells grown in ordinary media and which contain free tryptophanase.

* Happold (1929). *Brit. J. exp. Path.*

† Dawson & Happold (1943). *Biochem. J.* **37**, 389.

The Effect of pH on the Solubility of Sulphonamides. By H. A. KREBS and J. C. SPEAKMAN

The problem of the change of solubility of a sulphonamide with pH lends itself to a general treatment in accordance with simple physico-chemical principles. It is shown that the approximate solubility of a sulphonamide in buffer solutions or in urines of different pH can be predicted from two constants, the solubility of the undissociated sulphonamide (S^o) and the acid dissociation constant (K_a) of the compound. For physiological purposes most sulphonamides can be regarded as monobasic acids whose undissociated form is sparingly soluble but whose alkali salts are readily soluble. It follows from the equation for the electrolytic dissociation of this type of substance that $S = S^o(1 + 10^{pH-pK_a})$, where S is the solubility and pK_a represents the negative logarithm of the (classical) acid dissociation constant. Data published by Gilligan & Plummer* and our own measurements of sulphadiazine agree well with the theory:

	S (mg. sulphadiazine in 100 ml. 25°)	
pH	Observed	Calculated (taking $S^o = 6{\cdot}16$, $pK_a = 6{\cdot}45$)
4·89	6·3	6·3
6·01	8·5	8·4
6·35	11·1	11·0
6·82	19·4	20·6
7·23	43·5	43·5
7·56	86	86
7·67	114	109
8·00	229	226

A method for the determination of S^o and K_a from solubility measurements is described and the values for the two constants for the more common sulphonamides are given:

Acid dissociation constants (pK_a) of sulphonamides and their N^4*-acetyl derivatives and solubilities of the undissociated compounds (S^o) at* 38°

Drug	pK_a (refers to classical dissociation constant; ionic strength 0·1 M)	S^o (mg./100 ml.)
Sulphadiazine	6·28	9·9
N^4-acetyl sulphadiazine	5·86	18·6
Sulphathiazole	7·10	92
N^4-acetyl sulphathiazole	6·81	7·1
Sulphamezathine	7·38	64
N^4-acetyl sulphamezathine	7·14	84
Sulphamerazine	6·95	41
N^4-acetyl sulphamerazine	6·55	80
Sulphapyridine	8·32	50
N^4-acetyl sulphapyridine	8·02	31

Dissociation constants of drugs are liable to affect a variety of pharmacological properties. The non-absorption, for example, of the drugs of the sulphaguanidine and sulphasuxidine groups appears to be connected with non-ionization of the sulphonamide nitrogen. In sulphaguanidine both H atoms of the $—SO_2NH_2$ group are substituted and the acidic properties of this group are therefore abolished. In sulphasuxidine and sulphathalidine the acidic properties of the $—SO_2NH—$ group are so weakened by the introduction of another acidic radical into the molecule that they do not come into play under physiological conditions.

* Gilligan, D. R. & Plummer, N. (1943). *Proc. Soc. exp. Biol., N.Y.*, **53**, 142.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **244th Meeting** *of the Biochemical Society was held in the Human Nutrition Research Unit of the Medical Research Council at the National Hospital for Diseases of the Nervous System, Queen Square, London, W.C.* 1, *on Friday,* 28 *September* 1945, *at* 11 *a.m., when the following communications were given:*

COMMUNICATIONS

Faecal Excretion of Urobilinogen after Fractures. By N. F. Maclagan

In the course of work on a simplified method for estimating faecal urobilinogen, to be reported elsewhere, patients selected as 'normal' controls included a number with recent fractures of long-bones. These were found to give results well above those of the other control subjects, often reaching levels 2–3 times those finally accepted as normal. Thus the results in 11 cases were: 660, 584, 572, 515, 470, 466, 400, 295, 268, 223 and 210 mg. urobilinogen/100 g. wet stool (normal 30–220).

One case of fractured femur in a male adult was selected for quantitative study, and was found to excrete during the first 32 days after injury 5·67 g. of faecal urobilinogen in excess of his basic level, as estimated over a 12-day period 3 months later. This would suggest the haemolysis of a minimum of 142 g. of haemoglobin in excess of normal, corresponding in this patient to 1270 ml. of blood (Hb 80% Haldane). This volume of blood would appear to be considerably in excess of that present in the haematoma at the site of fracture, although absorption of this haematoma must contribute to the results. The alternative hypothesis of non-utilization of bile pigment also has to be considered.

It is thought that these observations may have some bearing on the etiology of traumatic anaemia, a condition in which the possibility of excessive haemolysis has already been suggested by Vaughan* on haematological grounds.

* Vaughan, J. M. (1945). Personal communication.

The Nitrogen Content of Human Total Plasma Proteins. By R. P. Cook

In estimations of total plasma proteins based on the Kjeldahl method the factor 6·25 is commonly used to convert N to protein on the assumption that the combined plasma proteins contain 16% N. It was to test this assumption that the following experiments were carried out.

Four 'pools' of plasma, using potassium oxalate as anticoagulant, from 67 supposedly normal persons were formed. The total heat-coagulable proteins were prepared by precipitating and washing the proteins with acetone, coagulating by heat in 0·6% saline at pH 5·0, washing with water, followed by alcohol and ether.

The proteins were analyzed for N by the Dumas method (Weiler & Strauss) and micro-Kjeldahl method, all precautions given by Chibnall, Rees & Williams* being adopted. The results for total plasma proteins and for the 'globulin' fraction (precipitation with saturated Na_2SO_4 solution), corrected for moisture and ash, are as follows:

	N % (corrected for moisture and ash)					
	No. of determinations		Dumas N	No. of determinations		Micro-Kjeldahl N
Total coagulable plasma proteins (4 pools)	8	Range	13·29–15·96	6	Range	14·71–15·54
		Mean	15·20±0·84		Mean	15·23±0·50
'Globulin' (3 pools)	4	Range	13·99–14·40	4	Range	13·45–15·13
		Mean	14·19		Mean	14·44

A comparison has also been made of the estimations based on the gravimetric method† with those by the Kjeldahl method. The results of eight determinations on the four pools are:

		Values as g. per 100 ml. plasma	
		Range	Mean
Total N	(*A*)	1·095–1·169	1·119±0·038
N.P.N. (Folin-Wu filtrate)	(*B*)	0·016–0·036	0·024±0·0067
'Protein' N	(*A*–*B*)	1·056–1·133	1·087±0·044
Gravimetric determination	(*C*)	6·90 –7·72	7·35 ±0·37
Total N × 6·25	(*D*)	6·87 –7·31	7·00 ±0·18
'Protein' N × 6·25	(*E*)	6·60 –7·08	6·79 ±0·30
'Protein' N × 6·5	(*F*)	6·86 –7·36	7·06 ±0·20

Fair agreement between the two methods is obtained if total N is multiplied by 6·25. Thus the mean value for $D/C \times 100$ is $95{\cdot}2 \pm 1{\cdot}14$. If 'protein' N is taken a higher factor should be used. For example, using 6·5 as factor the mean value for $F/C \times 100$ is $96{\cdot}1 \pm 1{\cdot}58$.

These results are at variance with those of Robinson & Hogden.‡ An explanation may be that in their determinations some hydrolysis took place during the coagulation process. The N content of the washings from their estimations was 64 mg./100 ml. plasma and gave a positive biuret reaction. In three of the above determinations values of 24, 58 and 31 mg. N/100 ml. plasma were found in the washings which gave a slight biuret reaction in one case only.

Very divergent values for the N content of plasma proteins either mixed or 'pure' are given in the literature. As total plasma proteins are hardly to be considered a definite and constant chemical species the best method of expression would be as g. N/100 ml. plasma.§

* Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
† Guillaumin, C. O., Wahl, R. & Laurencin, M. L. (1929). *Bull. Soc. Chim. biol., Paris*, **11**, 387.
‡ Robinson, H. W. & Hogden, C. G. (1941). *J. biol. Chem.* **140**, 853.
§ Cook, R. P., Keay, D. M. & McIntosh, D. G. (1945). *Brit. med. J.* (in the Press).

The Colorimetric Estimation of Synthetic Oestrogens in Pure Solutions with Special Reference to the Relative Stabilities of Stilboestrol and of Dienoestrol.

By F. L. Warren and F. Goulden

Investigations have been carried out on the methods proposed for chemical assay of synthetic oestrogens by Dingemanse* (development of a red colour by $SbCl_5$) and by Malpress† (measurement of the yellow colour obtained by nitration followed by neutralization). The results and conclusions obtained are summarized below.

Dingemanse's $SbCl_5$ *reaction.* Treatment of 1 ml. of alcohol-free chloroform solution of the oestrogen with 9·0 ml. of a solution of $SbCl_5$ in ethylene dichloride (5 ml. $SbCl_5$ diluted to 100 ml.) gives a reproducible red colour with stilboestrol and with dienoestrol. Weight for weight, stilboestrol gives the greater colour. With dienoestrol, maximum colour development occurs immediately on mixing; with stilboestrol the colour increases during the first 10 min. and then reaches a maximum. The Ilford Green Spectrum Filter gives maximum absorptions. Over the usual range of readings on a 'Spekker' photoelectric absorptiometer the calibration curves for both oestrogens are straight lines. At a concentration of 2·0 μg./ml. readings are 0·325 (stilboestrol) and 0·245 (dienoestrol).

Malpress's nitro-reaction. The following observations were made:

(*a*) The yellow colour is produced by general absorption at the violet end of the spectrum.

(*b*) The blue-green filter gives the highest values and the most sensitive slope to the calibration curve.

(*c*) The estimation is about 1/20 as sensitive as the $SbCl_5$ method.

Stability of stilboestrol in chloroform solution. Solutions of stilboestrol in alcohol-free chloroform were kept in clear-glass flasks in the light at room temperature. Daily measurements of the colour reactions ($SbCl_5$ and Malpress) showed no change over a period of 8 days. The solutions remained colourless.

Stability of dienoestrol in chloroform solution. With this oestrogen the $SbCl_5$ colour decreased as the solution aged, rapidly at first and then more slowly. As percentages of the initial colour the values were: 3 days, 55%; 6 days, 30%; 9 days, 20%; 16 days, 15%. Simultaneous determinations by Malpress's method showed a similar decrease in the colour developed. The decrease was not so rapid or so great as with the $SbCl_5$ values. The lowest value (16 days) was 53·5% of the initial. Similar observations were made in other solvents. All solutions so far encountered develop a yellow colour which gradually increases in intensity on standing.

The possibility that the 'decay' of dienoestrol solutions on ageing is brought about by (*a*) formation of diethylstilbene quinone or (*b*) polymerization, is being examined.

* Dingemanse, E. (1939). *Acta brev. neerl. Physiol.* **9**, 118.
† Malpress, F. H. (1945). *Biochem. J.* **39**, 95.

Availability of Histidine Derivatives from Growth. By A. Neuberger and T. A. Webster

The Isolation of a New Sulphuric Acid Ester from the Urine of Pregnant Mares. By W. Klyne and G. F. Marrian

Schachter & Marrian,* while isolating oestrone sulphate from mares' pregnancy urine, isolated a second crystalline substance which subsequent unpublished work showed to be a sulphuric acid ester, probably of steroid nature. The same, or a somewhat similar ester, has now been isolated from the same source by a modification of the methods previously used and has been more fully investigated.

The new steps in the method of purification are: (i) precipitation of the esters as potassium salts by half-saturation of an aqueous solution with potassium acetate; (ii) separation of a fraction sparingly soluble in water; and (iii) final purification and characterization of the ester as its *p*-toluidine salt.† From urine collected during the last month of pregnancy the new ester has been obtained in a yield of about 15 mg./l.

The *p*-toluidine salt forms fine white needles, m.p. 195–197° (corr.), $[\alpha]_D^{19°} = +32$ (3% in $CHCl_3$), and has the probable formula $(C_{21}H_{31}O.SO_4)^-$ $(CH_3C_6H_4NH_3)^+$. The potassium salt appears to exist in two different forms; one of m.p. 244–245° (dec., corr.) seems to be a monohydrate of the probable formula $(C_{21}H_{31}O.SO_4)^-.K^+.H_2O$. The solubility of the potassium salt in cold water is only 3 mg./ml.

The *p*-toluidine salt on acid hydrolysis yields a compound of the probable formula $C_{21}H_{32}O_2$, which melts at 205–207° (corr.). This compound is precipitable from 90% ethanol with digitonin and is therefore presumably a steroid, probably with a 3(*β*)-hydroxyl group. It yields a monoacetate, m.p. 162–164° (corr.). On treatment with semi-carbazide hydrochloride and sodium acetate no semi-carbazone is formed; in the Zimmermann test, however, a purple colour develops, but more slowly than with the 17-ketosteroids. The compound gives no colour with tetranitromethane, but since *αβ*-unsaturated ketosteroids such as cholestenone and progesterone give no colour with this reagent this is not conclusive evidence against the presence of an olefinic linkage.

The hydrolysis product of the ester appears to be non-identical with any compound hitherto isolated from mares' urine. Its composition, chemical properties, melting-point,

and the melting-point of its acetate suggest that it may be identical with Δ^{16}-*allo*-pregnene-3(β)-ol-20-one, which was obtained by Marker & Turner‡ from dihydro-*pseudo*-tigonenin diacetate.

* Schachter & Marrian (1938). *J. biol. Chem.* **126**, 663.
† Barton & Young (1943). *J. Amer. chem. Soc.* **65**, 294.
‡ Marker & Turner (1940). *J. Amer. chem. Soc.* **62**, 3003.

Desalting Solutions of Amino-acids. By R. Consden, A. H. Gordon and A. J. P. Martin

The apparatus consists of a carbon anode immersed in N/10-H_2SO_4 in a hanging bag of thick cellophane. The latter dips into the solution to be desalted which floats on a mercury cathode in a celluloid chamber. The mercury is circulated by a water lift pump. The passage of current discharges any metallic ions present, which dissolve into the mercury. The pumping water also serves to free the mercury from alkali metals. The anion of the salt passes through the membrane and accumulates in the anode solution. By employing a negative membrane such as cellophane the electroendosmosis is towards the cathode causing an increase in the time required for desalting but also helping to prevent the passage of neutral molecules such as the amino-acids into the anode solution. Even with the dicarboxylic amino-acids only negligible amounts pass into the anode solution which is sufficiently acid to reverse their charge. It is, of course, impossible to remove acids of strength comparable to that of the basic groups of the amino-acids. Thus with borax the Na^+ is removed as usual but boric acid remains with the amino-acids. Since neutral molecules are carried by electroendosmosis through the membrane away from the anode, the amino-acid solution may become contaminated with free halogens when the salts are halides unless special precautions are observed (e.g. replacement of the anode solution). With sulphates, however, no persulphate could be detected. In removing NaOH little H_2 is evolved, 1 ml. mol. was removed by 116 coulombs. 100% current efficiency would require 96·5 coulombs. In removing Na_2SO_4 the current efficiency is only 10–15%, i.e. the transport number of the $SO_4^=$ in the membrane, and the pH of the amino-acid solution falls until H_2 is evolved and the current efficiency for the removal of Na^+ by the mercury reaches the same figure. By partially replacing the H^+ ions in the anode solution by Na^+ ions the transport number of the $SO_4^=$ is increased and the current efficiency of desalting is correspondingly increased. The current eventually falls sharply to a constant value at which diffusion is balanced by electrical transport. Thus to minimize the final concentration of H_2SO_4 in the amino-acid solution the membrane should be thick and small in area. The potential gradient should be high and the H_2SO_4 in the anode solution should be dilute. The apparatus can dissipate 150 W., the voltage being finally raised to 300.

Nicotinic Acid in Unmalted and Malted Cereals and Other Foods. By E. M. James, F. W. Norris, J. G. Organ and F. Wokes

A comparative study of different chemical methods, using the Spekker absorptiometer for photoelectric estimation of the yellow colour given by nicotinic acid with cyanogen bromide and various amines, showed that

(*a*) *The colour intensity* for a given amount of nicotinic acid was in the ratio of *p*-aminoacetophenone (PAAP) 6; procaine 4; metol 1; working under optimal conditions.

(*b*) *The rate of colour formation*, measured by number of minutes taken to reach the maximum intensity, was in the following order of increasing time: PAAP+aqueous

solution; procaine with 6% HCl; procaine with 10% HCl; PAAP alcoholic solution; metol.

(*c*) *The rate of fading of the colour* decreased with increase in the time taken to reach the maximum. The metol colour, however, appeared to be stable for at least several hours.

(*d*) *Deviations from Beer's law* were least with PAAP and greatest with metol.

(*e*) *The experimental error*, measured by the coefficient of variation, was lowest with PAAP and highest with metol.

(*f*) *Interfering factors* in certain foods (e.g. milk) caused the reading to increase steadily for many hours, more rapidly with PAAP+aqueous solution than with procaine using 10% HCl. The readings which agreed most closely with the results of microbiological assays were those obtained within a few minutes of mixing.

(*g*) *Interfering factors* in other foodstuffs (e.g. malt extract), again causing the readings to become too high, were traced to the use of $KMnO_4$ to decolorize the extracts.

(*h*) These interfering factors could be overcome by an adsorption technique, using a modification of Dann's method.* The material, after digestion with 1 in 3 of conc. HCl at 100°, is treated with Lloyd's reagent at a pH below 1, and the adsorbed nicotinic acid is eluted at a pH of about 11. Finely powdered lead nitrate is added to bring the pH to between 8 and 9, when decolorization is completed. The pH is then brought to $6{\cdot}5 \pm 0{\cdot}3$ with Na_3PO_4. The cyanogen bromide method, using PAAP, is applied to the colourless extract, with recovery experiments at two levels, and reagent and acid alcohol blanks. PAAP+aqueous solution is more satisfactory than PAAP+alcoholic solution with malted products, and usually produces the maximal colour in 1–3 min.

Provided the microbiological assay was valid on general grounds no significant difference was found in the microbiological results whether these were calculated by the single curve method or by the slope ratio method.

Previous workers† have reported the occurrence in cereals of substances which interfere with the estimation of nicotinic acid. The possibility arose that these substances might be affected by malting processes. The problem has been investigated by parallel chemical and microbiological estimations on a series of samples of barley before and after malting, and on malt extracts prepared from the malted barleys, as well as on other foods. The closest agreement between chemical and microbiological results was obtained using the modified Dann's method outlined in the previous paper, and this also gave the lowest coefficient of variation in replicate assays. Methods‡ using $KMnO_4$ and *iso*butanol to decolorize the extracts gave wider deviations from microbiological results and higher coefficients of variation. The higher degree of accuracy obtained with the modified Dann's method was not affected by malting processes which had considerably increased the amount of interfering colour. Chemical and microbiological methods agreed in showing an increase of 15–25% in the nicotinic acid content during malting, but in the preparation of malt extract from the malted barley any further increase was not significant.

* Dann, W. J. & Handler, P. (1941). *J. biol. Chem.* **140**, 201.

† Kodicek, E. (1940). *Biochem. J.* **34**, 724. Oser, B. L., Melnick, D. & Siegel, L. (1941). *Abstr. Div. Biol. Chem. Amer. Chem. Soc.*

‡ Kodicek, E. & Wang, Y. L. (1943). *Biochem. J.* **37**, 530.

Changes in the Chloride Concentration of Sweat with Acclimatization. By W. S. S. Ladell (introduced by B. S. Platt)

The chloride concentration in serial sweat samples from the arms of subjects working in a hot humid environment rises as the exposure continues. In some subjects the rise may be as much as from 0·3 to 0·6% in 160 min. This increase is independent of rises in rectal temperature and of changes in the rate of sweating. The sweat glands must do

osmotic work to excrete the sweat which is always hypotonic, in all instances, but less work is being done when a more concentrated sweat is excreted—hence this rise in chloride concentration with duration of sweating may be a manifestation of fatigue; further evidence of fatigue is that the rate of sweating falls off at the end of a long exposure to heat.

In a series of consecutive exposures (rapid acclimatization) of a number of subjects the chloride concentration of sweat from a given subject at a given time varied irregularly. If the total salt loss of the body is calculated from the sweat losses over each period and the chloride concentration of the arm sample collected during that period (evidence will shortly be published that this is a valid calculation), it is found that the total salt loss is practically constant in a given individual from day to day for a given total of sweat lost so long as the rate of sweating remains the same. Thus in rapid acclimatization there is no fall in sweat chloride concentration such as has been described by Dill and others.*

But when a subject lived in the hot room day and night at such day temperatures that he was sweating profusely for up to 8 hr. a day, after the first few days' sojourn the chloride concentration of his sweat no longer rose with time. This change, which amounts to a fall in chloride concentration when total losses are considered, was not due to a salt lack as the subject was not losing weight and plasma chloride level was normal. After leaving the hot room he lost this acclimatization effect and, on being re-exposed to the heat some weeks later, the expected rise in chloride concentration with time was seen again.

Subjects exposed to the heat for some hours every day over a period of many months show the same effect but less markedly. The table shows the salt losses from two men at various times during an 18-month period during which they were in continual use as

Mean salt losses of two subjects at various times

Subject	Period	No. in series	Mean rate of sweating g./min.	g. salt lost at sweat loss of 500 g.	1000 g.	2000 g.	3000 g.	4000 g.	5000 g.
PIK	May 1944	4	7·3	1·34	—	—	—	—	—
	June 1944	4	9·9	1·66	3·99	—	—	—	—
	Sept. 1944	8	12·6	1·91	4·23	—	—	—	—
	Dec. 1944	4	14·5	2·57	6·27	13·96	—	—	—
	Jan. 1945	6	8·8	2·78	5·98	—	—	—	—
	Feb. 1945	5	14·2	2·54	5·41	11·29	—	—	—
	Apr. 1945	6	14·9	2·31	4·79	—	—	—	—
	July 1945	8	18·4	2·31	4·91	11·50	15·12	—	—
	Aug. 1945	7	20·2	2·46	5·31	10·83	16·02	—	—
BRA	Sept. 1944	6	19·0	—	3·96	9·27	15·36*	—	—
	Feb. 1945	4	22·3	—	3·70	8·40	13·03	20·78*	—
	Apr. 1945	6	20·4	—	3·84	7·52	—	—	—
	July 1945	7	27·5	—	3·84	8·45	13·17	17·24	—
	Aug. 1945	10	31·4	—	3·62	7·99	12·47	16·57	21·02

* Indicates only one observation.

experimental subjects in the hot room. The salt losses for small losses of sweat (1–2 l.) has not varied much during this period, but the salt losses for heavy sweating have become less, showing once more the diminished liability of the sweat glands to fatigue.

This diminution in the salt losses from the sweat which occurs with hyper-acclimatization is not to be confused with the effect of salt deficiency. McCance† showed that in severe salt deficiency there is a fall in the chloride concentration of sweat; in the present series of experiments a diminution of the salt intake, even though short of that needed to give rise to salt deficiency, resulted in a fall in the chloride concentration of sweat.

* Dill, D. B., Hall, F. C. & Edwards, H. P. (1938). *Amer. J. Physiol.* **123**, 412.

† McCance, R. A. (1938). *J. Physiol.* **92**, 208.

The Chemical Micro-biology of Kaffir Beer. By R. A. WEBB (introduced by B. S. PLATT)

The technique employed by native Africans in brewing beer from cereal grains resembles, superficially, that employed in European brewing. The grain is soaked in water and germinated, then dried, ground, and mashed in warm water.

When fermentable sugar is available the bacteria and yeasts introduced fortuitously with the malt and meal carry out lactic and alcoholic fermentations. The greater part of the lactic fermentation takes place in the first 24 hr. Alcoholic fermentation begins after about 24 hr. and continues usually for 4 days. The beer may be consumed after the first day, when it is sweet and acid, or after 5 days, when it is alcoholic and acid. The average alcohol content is 3%, although values up to 8% have been recorded. If left unconsumed after the fifth day acetic fermentation begins and gradually renders the beer unpalatable.

Early in the present investigation it was found that the starch saccharifying power of germinated maize was very low and recently Kneen* has shown that germinated maize and sorghum, which are widely used in the preparation of Kaffir beer, have only one-twentieth the saccharifying power of barley malt. The germinated grain is not, in itself, sufficient to account for the sugar production necessary to achieve the alcohol contents found in practice.

Apart from bacteria and yeasts, 22 species of microfungi comprised the microflora of 19 samples of brewing materials examined. Of these species *Aspergillus flavus* occurred, often in very large numbers, in 14 of the samples, and a species of *Mucor* in 5 others. Both these species are capable of carrying out extensive starch hydrolysis. In a 10% cooked and sterilized mash of maize meal a pure culture of *Aspergillus flavus* produced over 5% sugar (as glucose) in 19 hr. and 8·6% in 96 hr. A beer brewed from maize malt which was almost free from *A. flavus* reached only 1·2% alcohol, while the same malt with the addition of a culture of the fungus reached 3·2% alcohol in the same time.

The recognition of the role of *A. flavus* or *Mucor* sp. reported by Doidge† solves one of the problems encountered by others investigating Kaffir beer (cf. Lazar).‡

The nutritional value of the beer, which is consumed unstrained, is under investigation. A general account has been given§ already indicating the part played by Kaffir beer in human nutrition.

* Kneen, E. (1944). *Cereal Chemistry*, **21**, 304–13.

† Doidge, E. M. (1910). *Transv. Dep. Agric. Sci. Bull.* no. 5.

‡ Lazar, O. (1938). *J. S. Afr. Chem. Inst.* **21**, 23–32.

§ Platt, B. S. & Webb, R. A. (1945). *Biochem. J.* **39**, *Proc.* xxi and *Proc. Nutrit. Soc.*—full paper (in the Press).

A Critical Examination of the Estimation of Methionine by H_2O_2 Oxidation. By C. R. C. HEARD (introduced by B. S. PLATT)

Albanese's* method for the estimation of methionine in urine or protein hydrolysates depends on selective oxidation by H_2O_2 and iodometric titration of excess H_2O_2. He claimed that 0·025 M-H_2O_2, 0·5 M-$HClO_4$ (as a catalyst), a temperature of 20–25° C. and a reaction time of 60 min. gave 100% oxidation of methionine (5–40 mg. in 20 ml. total volume), and that exceeding the prescribed concentrations, temperature or time caused 'over-oxidation' of methionine and interference from cystine or tryptophan. A careful study of all these variables reveals that some of the dangers have been exaggerated, while others were ignored.

In 60 min. 0·025 M-H_2O_2 gave only 85% oxidation of methionine, and either 0·03–0·04 M-H_2O_2 or considerably increased time was necessary for complete oxidation. There

was no 'over-oxidation' of methionine with up to 0·04 M-H_2O_2 and times up to 18 hr., nor did cystine interfere. Tryptophan, however, was 20 % oxidized by 0·03 M and 100 % by 0·04 M-H_2O_2; when the H_2O_2 concentration was restricted to 0·025 M-H_2O_2 there was no oxidation of tryptophan within 4 hr. The choice of time seemed quite arbitrary, provided it was kept the same for all samples in the same experiment. At temperatures from 0 to 55° C. there was no oxidation of cystine or of tryptophan in 60 min.; with increased temperature, methionine was completely oxidized without sign of 'over-oxidation'. Perchloric acid (0·5 M) slightly accelerated the oxidation of methionine without affecting cystine or tryptophan under any of the above conditions.

In the presence during oxidation of a final concentration of 2 N-HCl there was 10 % 'over-oxidation' of methionine, 12 % oxidation of tryptophan and 50 % oxidation of cystine. The magnitude of the error was related to HCl concentration. If the HCl was neutralized before adding H_2O_2, etc., there was no 'over-oxidation' of methionine and no oxidation of tryptophan in 60 min., but cystine was still 21 % oxidized. If, however, methionine was present with cystine in equivalent concentration, the values obtained were the same as for methionine alone, presumably because the more reactive methionine reduced the H_2O_2 concentration rapidly to a point where cystine was not appreciably oxidized. If HCl is present in an unknown solution, its effect must be determined and controlled by neutralization and by adding methionine and cystine to additional aliquots of the unknown before oxidation.

Calculation of the methionine content of an unknown sample is made by comparison with an identically treated standard (since with 0·025 M-H_2O_2 at 25° C. the reaction is incomplete in 60 min.). It was found essential to have the molar concentration of methionine no greater than half that of the H_2O_2 if a lineal relationship was to be established between methionine and its equivalent in N/10-thiosulphate, and that preferably unknown and standard solutions should contain nearly the same amount of methionine.

* Albanese, A. A., Frankston, J. E. & Irby, V. (1944). *J. biol. Chem.* **156**, 293.

Determination of Sulphur in Biological Material by Hydrogenation. By June Olley (introduced by B. S. Platt)

The method is essentially that of ter Meulen* modified by Masters† for biological material. It depends on the reduction of S to H_2S with platinized asbestos as catalyst; the H_2S is precipitated as cadmium sulphide and estimated iodimetrically. The advantage of hydrogenation over the generally used methods based on oxidation are speed, smaller size of samples and prevention of loss of S by volatilization.

The combustion tube is heated electrically. The heating coils are wound on a porcelain combustion tube,‡ 60 cm. long and 18 mm. internal diameter. The heating coils are in six sections, arranged so that they can be turned on separately, with balancing resistances in parallel so that the total resistance of the circuit remains at 50 ohms (230 V.). In order to obtain a sufficiently high temperature, it is important to lag well with asbestos string. The quartz combustion tube§ is inserted into the 'furnace'. Ground-glass cones, B24 standard, are attached to both ends. One socket is pulled out to attach to the bubbler and the other bent at right angles to dip into the $CdCl_2$ solution. Hydrogen is passed directly from a cylinder provided with a safety trap.

Theoretical results were obtained for S in methionine and cystine with this arrangement. But in the estimation of S in casein with regeneration of the catalyst with air, very variable results were obtained and in some runs no H_2S was produced. When, however, the catalyst was regenerated by heating the tube in a stream of oxygen, consistent but still low values were obtained. The casein analyzed was found to contain appreciable quantities of Na salts as impurity; so HCl was added to the sample (Gauthier),‖ but the tube cracked after two or three estimations.

The final procedure adopted with all biological materials in view of the possible presence of alkali metals was to regenerate the catalyst with oxygen between each estimation and hydrogenate in the presence of hydrogen chloride. This condition was obtained by introducing a second bubbler containing concentrated hydrochloric acid between the combustion tube and the NaOH bubbler. This stream of HCl also eliminated the necessity for cleaning the tube with HCl after several estimations.

It was intended to estimate very small quantities of S by precipitation as PbS and determination of the Pb colorimetrically, but hydrogenation in HCl caused $PbCl_2$ to be precipitated. It was found, however, that duplicates to within 2% could be obtained by iodimetric titration with a microburette for quantities as low as 0·05 mg. S.

Substance	% S of dry wt.	
Cystine	26·55	Theoretical 26·67%
Methionine	21·40	Theoretical 21·50%
Casein (impure)	0·33	Without HCl; catalyst regenerated with oxygen
Casein (impure)	0·523	With HCl; catalyst regenerated with oxygen
Casein (impure)	0·522	Sodium peroxide fusion
Rat hair (fat extracted)	3·05	
Collagen from rat skin	0·261	
Total rat carcass without pelt	0·69	

* ter Meulen (1922). *Rec. Trav. Chim. Pays-Bas*, **41**, 112.
† Masters (1939). *Biochem. J.* **33**, 1318.
‡ Obtainable from Morgan Crucible Company.
§ Obtainable from Thermal Syndicate.
‖ Gauthier (1935). *Bull. Soc. Chim.* **2**, 322.

Differential Adsorption of Pepsin and Rennin on Foam. By G. Andrews and F. Schütz

Adsorption on foam, by means of a technique described by one of us,* was recently found useful, at one state, in the purification of serum choline esterase.† Impurities were removed by foam at a pH where the enzyme was little adsorbed. At another pH the enzyme was adsorbed, but destroyed. We have now found that pepsin and rennin are not destroyed in this way.

The relative adsorption on foam of these enzymes has been studied, not so much to find a new way of purification, but to show that by investigations of this kind differences in the surface activities of enzymes, present in the same solution, can be detected. Crystalline pepsin is known to have a high rennin activity, and the question whether both activities are due to the same, or two different enzymes has been the subject of a controversy. It seems that this has now been settled, especially by the recent work of Berridge.‡

pH 1·68–2·0, a concentration of 0·4% in 0·01 M-citrate buffer, and a temperature of 30° were found optimal for foaming of commercial pepsin (B.D.H.). The foaming was carried out by introducing nitrogen at a low rate into the apparatus as previously described. The gas jet had an opening of 50 μ diameter. The pepsin activity was determined according to Michaelis & Rothstein,§ rennin activity according to Hankinson & Palmer.‖

If the ratio of pepsin : rennin activity per dry weight of the original solution was called 1 : 1, it was 3·3 ($\pm$0·3) : 1·3 ($\pm$0·1) in the first foam fraction, and, if this was foamed again, it was 7·4 : 2·6. Thus in a simple way a preparation of pepsin was obtained which was 7–8 times purer than the original powder, with a relative reduction of rennin activity of 60–68%. Destruction was excluded as the cause of the relatively lower rennin activity, because the solution which remained behind, after removal of the

foam fraction, showed perfect agreement with the calculated rennin activity (original solution minus foam fraction).

It must, therefore, be concluded that, under the above conditions, pepsin is considerably more surface active than rennin.

* Schütz, F. (1937). *Nature, Lond.*, **139**, 629. (1942). *Trans. Faraday Soc.* **38**, 85 and 94; and in the Press.

† Bader, R., Schütz, F. & Stacey, M. (1944). *Nature, Lond.*, **154**, 183.

‡ Berridge, N. J. (1945). *Biochem. J.* **39**, 179.

§ Michaelis, L. & Rothstein, L. (1920). *Biochem. Z.* **105**, 60.

‖ Hankinson, C. L. & Palmer, L. S. (1942). *J. Dairy Sci.* **25**, 277.

DEMONSTRATIONS

1. **Materials and Method used in the Brewing of Kaffir Beer.** By R. A. Webb (introduced by B. S. Platt)

2. **Drying Food Samples by the Solvent Method, using Isopropyl Ether.** By M. W. Grant and B. S. Platt

3. **The Estimation of Sulphur by Hydrogenation.** By June Olley (introduced by B. S. Platt)

4. **The Dehydration of Foodstuffs in Edible Oils under Vacuum.** By B. S. Platt and members of the Human Nutrition Research Unit of the Medical Research Council

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The **245th Meeting** *of the Biochemical Society was held in the London School of Hygiene, Keppel Street, London, W.C.* 1, *on Saturday,* 10 *November* 1945 *at* 11.30 *a.m., when a discussion took place on 'The Chemical Basis of Cell Structure and Function', and the following communications were given:*

The Permeability of Cells. By J. F. Danielli

Under this heading must be considered (1) the plasma membrane, (2) the nuclear membrane, (3) the cytoplasm, (4) the nucleoplasm, (5) vacuoles, granules, mitochondria, etc.

The cell plasma membrane usually consists of a lipoid layer about 50 A. in thickness, adsorbed on to the surfaces of which is at least a monolayer of protein. This membrane controls the rate at which substances diffuse into and out of cells. This communication will deal almost exclusively with this plasma membrane. Its permeability is determined almost entirely by the lipoid layer.

The nuclear membrane has not been intensively studied: this is probably because of the experimental difficulties. It is known to have a permeability to water not greatly less than that of the plasma membrane. Its permeability to dyes appears to be low. It is known to be permeable to phosphate and some amino-acids: the magnitude of the permeability to these substances is unknown, but in some cases a lower limit to the permeability can be set.

The permeability of cytoplasm has been investigated by micro-injection of dyes and by electrical conductivity studies. Apart from the included bodies (granules, etc.), it appears to be freely permeable to molecules as large as trypan blue, and possibly to larger ones. The viscous resistance offered to movement of small molecules and small ions is not much greater than that of water.

The permeability of the nucleoplasm has not so far been successfully studied. Determinations of the viscosity of cell nuclei yield values greater than that of water, but not vastly greater. It is therefore probable that the nucleoplasm is relatively permeable.

The permeability of the vacuoles, mitochondria, granules, etc. is unknown. It is likely that in some cases the permeability of these included bodies is very low.

The remainder of this communication will deal with the physico-chemical mechanisms determining the permeability of the plasma membrane. This permeability can only be determined from studies of the rate at which molecules move across the membrane under known conditions. For example, the fact that a normal muscle fibre contains little sodium and little chloride tells us only that the dynamic *equilibrium* set up under normal conditions is such that a low concentration of these ions is reached in the cell: it tells us nothing of the *rate* at which ions move into and out of the cell.

Of the cells which have been studied in sufficient detail, all except those of the sulphur bacterium, *Beggiatoa mirabilis*, have a plasma membrane whose permeability is determined by a lipoid layer. Molecules diffusing through porous membranes obey the relationship $PM^{\frac{1}{2}}$ = constant, if the pore diameter is large compared with the diameters of the diffusing molecules (P = permeability, M = molecular weight). This relationship is obeyed by molecules diffusing into *Beggiatoa* for molecules up to the size of erythritol, but at the size of sucrose the permeability is much lower than predicted, so that the average pore diameter is of the order of that of sucrose. Thus with *Beggiatoa* the factor causing selective permeation is the relatively simple one of pore diameter.

With other cell membranes the situation is more complicated. When a molecule passes from an aqueous phase into the lipoid layer, it must first break free from the hydrogen bond and the Van der Waals forces acting between itself and water, and also make a hole in the lipoid layer in which it may be contained. The amount of energy involved in this may be large; with a molecule forming as many or more hydrogen bonds with water as does erythritol the energy may be 20,000 kg.cal. or more per g.mol., and penetration is slow. In such a case the main site of resistance to entry into the cell is at the lipoid-water interface itself, i.e. with molecules containing as many or more polar groups as erythritol it is the rate of passage through the lipoid-water interface which determines the rate of permeation. Molecules for which this is true obey the relationship

$$PM^{\frac{1}{2}} \exp [2500x/RT]/B = \text{a constant}$$

(x = number of unscreened CH and CH_2 groups in the molecule, R = gas constant, T = absolute temperature, B = lipoid-water partition coefficient).

Small molecules having few polar groups pass into the lipoid layer relatively easily. This lipoid layer, being composed of molecules such as tripalmitin, cholesterol and lecithin, has a relatively high viscosity, so that the main resistance to penetration of such molecules is the viscous resistance of the lipoid layer. These small non-polar molecules penetrate rapidly, obeying the relationship $PM^{\frac{1}{2}}/B$ = constant.

Large polar molecules, such as proteins, and large non-polar molecules, such as cholesterol, cannot penetrate a thin lipoid membrane by simple diffusion, and special mechanisms must be involved in their movements into and out of cells. There is also evidence that a small fraction of the area of some cells is differentiated to permit the passage of particular molecular types, such as glycerol or glucose. These special areas in some cases resemble the active patches of catalysts in that their special function can be destroyed by small amounts of poisons.

It is important to notice that different cells may differ widely in their permeability to the same molecular species. A variation of one hundredfold is common, and of ten thousandfold is known.

The problems in the field of cell permeability most urgently needing solution are the permeability of the nuclear membrane, the mechanisms of penetration of lipoids and proteins, and the nature of the active patches referred to above. The problem of secretion is related to these problems, but is more complicated in that a source of supply of energy is also needed.

The Physical Chemistry of Lipoid Protein Associations. By J. H. SCHULMAN

The significance of lipoid and protein associations in cell function and structure has long been known, but the meaning of these associations in terms of physical chemistry is still very vague. This may be due to the fact that there are few quantitative methods available to measure these associations, and further, the interpretation of these observations in terms of biology is very difficult.

Recent advances in surface chemistry and emulsion techniques have helped to clarify some of these by enabling quantitative data to be obtained. A very important point in considering these associations is the state of dispersion of the lipoid, i.e. whether it is molecular or aggregated. If the lipoid presents an interface, completely different results will be obtained from those obtained if it acts as a dispersant or adsorbent on to the protein.

For example, if in aggregate form as a suspension or in monolayer form at the oil/water interface, the protein molecule will unfold if association takes place. The unfolding *in vivo* is probably readily reversible to the native form, but *in vitro* the reversal takes many forms from native to various degrees of denaturation according to the history of the molecule in its unrolled state. Recent work of Frazer, Elkes & Schulman has shown that the protein molecule will associate with a lipoid molecule at an interface in a well-defined stoichiometrical ratio, when (confirming the work of Neurath & Putnam) the molecule is dispersed a whole range of complexes can be established. The measurement of associations with pure lipoids or lipins in aggregate form as at an interface, i.e. monolayer or emulsion or suspension, with monodisperse proteins can be classified reasonably on simple colloid principles. When the associating proteins are polydisperse and have at the same time certain lipoids already associating with them, as, for example, in serum and other biological plasmas or saps, etc., a competition and exchange can take place. The reversibility of the lipoid associating with the protein in its globular form or in its stretched or unrolled form, and the competition of a third body either protein or lipoid with the two principal associating compounds, together with the reversibility of the two forms of the protein molecule are concepts for which quantitative data are required.

*The emulsion technique**

This technique is especially applicable where both associating components are readily water-soluble. The lipin is adsorbed at the oil/water interface, and sufficient knowledge is available to regulate the interfacial area and concentration of the lipin at the interface and in bulk solution. Monodisperse proteins will unfold at the charged interface according to their isoelectric point and the sign of the charge on the oil/water interface. Thus below their isoelectric point proteins are positive and will associate with anionic soaps at the interface; above their isoelectric they will associate with cationic soaps at the oil/water interface. The flocculation of the emulsion takes place sharply at the coherent monolayer concentration of unrolled protein. This concentration at a saturated lipin interface is equivalent to the concentration of a protein monolayer at an uncharged oil

or air/water interface. At lower concentrations of lipin at the interface, the protein necessary for flocculation of the emulsion is proportional to the lipin concentration at the interface. If the lipin is free in solution a zone of flocculation is obtained, under certain conditions, showing varying ranges of lipin protein association. The protein molecule if it has not absorbed on to the flocculation interface, can still be in its native state.†

Two interesting observations can be made with the emulsion technique: first that the protein can be readily desorbed back into solution again in a monodisperse form on changing the pH of the solution to the non-adsorption range. The structure of this desorbed protein in relation to its history in its monolayer form is proving of great interest. The second point is that adsorption on to positive or negative surfaces commences sharply with most proteins at their isoelectric point (such as haemoglobin, serum albumin, α, β-globulins), but with γ-globulin there is a large overlap of some 1·5 pH units, i.e. the γ-globulin molecule can adsorb against a surface field.

Monolayer penetration technique

This technique is very suitable for measuring associations of proteins with lipoids. It consists of spreading a film of the lipoid at the air/water interface and compressing the film to the pressure at which a protein film would on its own collapse, or if there was no association, be driven out of the monolayer. This surface pressure is 16 dynes/cm.

Very dilute solutions of proteins are then injected into the underlying solution (of the order 1 mg./100 c.c.) changes in surface pressure surface viscosity or rigidity and surface potential are measured. It can be shown that if penetration of the lipoid film takes place all three of the above criteria alter radically. Strong association between the lipoid film and the protein can be shown by rises of surface pressure from 16 to 30 dynes/cm. In collaboration with Miss N. M. Czeczowiczka this method was applied to a whole series of lipoid monolayers (C_{16} amine, C_{20} amine, kephalin, cholesterol, lecithin) at three different pH's, 3·7, 8·5 and 10. Into the solution underlying these films serums and serum proteins from various sources were injected, and their effect on the lipoid films observed by surface methods. The table gives preliminary results of the effects as observed with changes in surface pressure only.

Film	$C_{16}H_{33}NH_2$			$C_{20}H_{41}NH_2$		
Injected pH ...	3·7	8·5	10	3·7	8·5	10
Fresh horse serum	−	+ + +	+ + + +	+ + + +	+	+ +
Heated horse serum	+	+	+ + +	+ + + +	−	±
Horse-serum albumin	+ + +	+ + + +	+ + + + +	+ + + +	+ + +	+ + + + +
Horse-serum globulins	−	+ + +	+ + +	+ +	+ +	+ +
Human serum	+ +	+ + +	+ + + + +	+ + + +	+ + +	+ + +
Human-serum albumin	+ +	+ + + +	+ + + +	+ + +	+ + +	+ + +
Human-serum γ-globulin	−	+	+ + +	+	+	+

Film	Kephalin			Cholesterol		
Injected pH ...	3·7	8·5	10	3·7	8·5	10
Fresh horse serum	+ + +	−	−	+ + + + +	+ + + +	+ + + + +
Heated horse serum	+	−	−	+ + + + +	+ + + +	+ + + + +
Horse-serum albumin	+	−	−	+ + + + +	+ + + + +	+ + + + +
Horse-serum globulins	+	−	−	+ + + + +	+ + + +	+ + + + +
Human serum	+	−	−	+ + + + +	+ + + + +	+ + + + +
Human-serum albumin	+ + +	−	−	+ + + + +	+ + + + +	+ + + +
Human-serum γ-globulin	+	−	−	+ + + +	+ + + +	+ + + +

In lecithin there was no association.

−	fall in S.T. below 16 dynes/cm. or const. at 16 dynes/cm.
±	final S.T. between 16 and 17 dynes/cm.
+	final S.T. between 17 and 20 dynes/cm.
+ +	final S.T. between 20 and 22 dynes/cm.
+ + +	final S.T. between 22 and 25 dynes/cm.
+ + + +	final S.T. between 25 and 28 dynes/cm.
+ + + + +	final S.T. above 28 dynes/cm.

Cholesterol adsorbs all proteins non-specifically and very strongly. It has previously been shown that at surface pressures above 16 dynes/cm. the cholesterol protein association is in the form of a double film very fluid in viscosity, and that on release of the pressure at 16 dynes the film suddenly gels strongly, showing that the protein and lipoid molecules now form a mixed monolayer. This phenomenon occurred with an optimum when the ratio of protein to lipoid was 4 : 1.‡

Lecithin shows no significant change when protein is injected into the underlying solution except at pH's above 11 or below 2. It is interesting that lecithin only shows association with serum when the serum is pathological (such as luetic serum). This could be expected owing to the strong internal salt linkage between phosphoric acid ion and the quaternary substituted amine ion.

Kephalin showed very interesting properties. Owing to the weak amine base it is negatively charged in pH solutions above 2. It could therefore be expected to associate with proteins in solutions below their isoelectric point. It associates strongly with fresh serum but not with heated serum or in acid solutions. This contrasts very markedly with the long-chain amines which do not discriminate between heated and unheated serum.

Long-chain amines have a very interesting property in that their association with fresh horse serum varies markedly with the hydrocarbon chain length. Thus in acid solution C_{16} amine only associates in neutral or alkali pH, whereas the C_{20} amine does the reverse, only associating markedly in acid solution with fresh or heated horse serum and not in alkali solution. This peculiar specificity does not occur with the separated serum proteins in the same way. Albumin appears to be much more reactive than γ-globulin which does not associate with C_{16} amine in acid solution.

A possible explanation of these results could be given by the presence of a lipoid in the serum which competes with the protein molecule in its association with the surface amine. In other words there is competition between the surface lipoid and the dispersed lipoid for the protein molecule which is further complicated by a possible association between the two lipoids themselves. This method gives a new approach to the study of serum reactions taking place at surfaces.

* Elkes, Frazer, Schulman and Stewart (1945). *Proc. Roy. Soc.* A, **184**, 102.

† Putnam, F. W. & Neurath, H. (1944). *J. Amer. Chem. Soc.* **55**, 692. Elkes, J. J., Frazer, A. C. & Schulman, J. H. (1945). *J. Physiol.* **104**, 18 *P*.

‡ Schulman & Rideal (1937), *Proc. Roy. Soc.* A, **122**, 46.

The Structural Proteins of the Cell. By W. T. Astbury

The finer details of protein structure still remain to be worked out, but in the light of X-ray and related studies the broader outlines are now beginning to appear. The fundamental clues are given by investigations of the fibrous proteins, which are seen to fall into two principal families, the keratin-myosin-fibrinogen group and the collagen group, distinguished chiefly by the long-range elasticity of the former. In spite of considerable variations in chemical constitution within both groups, these two master plans are preserved throughout. In the k.m.f. group the unit of structure is a polypeptide grid whose normal equilibrium configuration is not flat, but buckled; that is, the main chains are thrown into a series of folds that lie in planes transverse to the cross-linkages of the grid; and the basis of the long-range elasticity is the capacity to unfold and refold. The essential structural problem is the nature of the intramolecular fold that characterizes the group, and accumulated evidence now points to a simple system of close packing of the side chains, the polars on one side of the main chain and the non-polars on the other. Such a plan is sufficient to explain the observed striking unity amidst chemical diversity, and in particular it offers against its proper molecular background the most satisfying

interpretation of muscular activity. The plan of the k.m.f. group is one of the basal ideas in vital architecture. It represents a fundamental power of the cell of synthesising elastic fibrous protein of a standard molecular form, yet whose details can be adapted to a variety of ends as the processes of differentiation demand.

By a generalization of the idea of chain folding first derived from the study of the fibrous proteins we arrive at the concept of the corpuscular proteins, and then at the interpretation of denaturation as a process of disorganization whereby the peptide chains are liberated from their specific configurations; and it has in fact been demonstrated conclusively by X-ray methods (leading to actual industrial processes) that artificial fibrous proteins can be spun, after denaturation, from many corpuscular proteins. By an extension of the idea of close packing of the side chains we arrive at the concept of the protein *lamina* or membrane, probably again usually with all or most of the polars on one side and the non-polars on the other; and the next step is to build up combinations of two or more laminae, sometimes alone and sometimes sandwiched with accessory groupings, such as lipoid laminae. It was an early deduction from X-ray and monolayer data that the egg albumin molecule consists of four superposed laminae of folded polypeptide chains, and recent physico-chemical results strongly support that suggestion. Also, the X-ray findings of Perutz and Boyes-Watson now point to a similar conclusion for the structure of the haemoglobin molecule. Furthermore, as pointed out by Dervichian and others, laminae formed by suitable systems of *hexagonal* close packing might conceivably provide the explanation of the 2's and 3's found exactly or approximately in the stoichiometric make-up of many proteins.

The fibrous proteins of the collagen group appear to be composed of polypeptide chains in an extended configuration that differs from the extended (*trans*) form of the k.m.f. group in that it is partly *cis* on account of a preponderance of the imino residues of proline and hydroxyproline; but the collagen fibres, too, pass rapidly into a folded state under the action of heat and/or swelling agents, and then they also show long-range elasticity. The molecular structural link between the k.m.f. group and the collagen group has not yet been found, though one might be expected on general grounds, and certain histological evidence indicates as much. It may be that the transition between the two lies by way of the so-called reticulin, recent X-ray photographs of which seem to show characteristics both of keratin and of collagen.

The full pattern along the chains in both the k.m.f. group and the collagen group is on a giant scale as molecules go, so great as to fall within the range of the electron microscope. In collagen it amounts by X-rays to about 640 A., and Schmitt and his co-workers have demonstrated in the finest fibrils under the electron microscope a corresponding system of light and dark bands. In certain muscle fibrils, with a similar high X-ray period, they have revealed more detail still after treatment with phosphotungstic acid. The impression one gets from combined X-ray and electron microscope studies is of 'patterns within patterns', of successive levels of organization proceeding from the basic plans revealed in large-angle X-ray photographs up to the relatively enormous fibrils, and thence to combinations of fibrils. In this progressive building-up process the primal mechanism is probably a molecular template action, and in the transition from one level to the next we possibly have to do with small proportions of non-protein components, e.g. nucleotides and carbohydrates. The suggestion is that it is now necessary to estimate such small-proportion components with much greater precision, to see if they are indeed in definite and structurally significant stoichiometric relation to the remainder.

The Chemistry of Cell Nuclei. By E. STEDMAN

Owing to the relative ease with which they can be isolated, the heads of fish sperm and the nuclei of avian erythrocytes have, until recently, formed the chief material for the investigation of the chemical nature of cell nuclei. Such investigations have led to the conclusion that, apart from lipoids and inorganic material, these nuclei are composed of two constituents: (1) a basic protein, which may be either a protamin or histone according to the species or type of nucleus examined, and (2) desoxyribosenucleic acid. Upon this chemical foundation, cytologists have formed the theory that chromosomes consist of protamin or histone nucleate, and many cytological experiments have been interpreted on this basis. A reinvestigation of the chemical nature of cell nuclei has, however, shown that the above view of their simple composition must be abandoned. The chief results of this investigation are described below; the consequences they have for the theory of the chemical nature of the nucleus are also discussed.

The nuclei examined have been derived from various cells, including fish sperm, bird and fish erythrocytes, liver cells from various species, thymocytes, lymphocytes, spleen cells, chick embryo cells and tumour cells of different types. They exhibited a remarkably uniform qualitative composition. All of them contained nucleic acid and a basic protein, but in no case did the combined weights of these two constituents account for the dry weight of the nuclei. Full analytical details are not in many instances yet available, but, in view of the earlier statements regarding the composition of the heads of herring sperm and the nuclei of fowl erythrocytes, it is of interest to note that the two components in question accounted for approximately 60 and 65 % respectively of the dry weights of these nuclei. The remaining material consists largely of a protein of acidic character, for which the name of chromosomin has been proposed. A similar protein has been detected in all the nuclei examined and it is therefore justifiable to conclude that nuclei consist, in general, of three main components: nucleic acid, basic protein and chromosomin.

With the probable exception of nucleic acid, which appears to be of invariable composition, these nuclear components differ in composition according to the species from which they are derived. This is obvious in the case of the basic protein, for it may consist of a protamin, as in salmon or herring sperm, or of a histone, as in cod sperm. In some cases, however, the basic protein also varies within the species, for, in the salmon, whereas the sperm contains a protamin, the nuclei of the somatic cells, including the sperm mother cells, contain histones. There is, moreover, evidence that the histone in the nuclei of the liver cells of this species differs in composition from that in the erythrocytes. It is not yet known if this result can be generalized. From their distribution it is clear that histones and protamins, which never occur together in the same nucleus, exercise similar physiological functions, and it is therefore doubtful if their classification into two groups is justified on biological grounds. Nevertheless, the distinction is convenient from a chemical standpoint, for the two best known protamins (salmin, clupein) differ from histones in containing no aromatic amino acids. Histones can therefore be readily distinguished from these protamins by their content of tyrosine. Histones have also been stated to contain tryptophan. This, however, is entirely contrary to our experience; unless contaminated with other proteins they give no detectable tryptophan reaction. This absence of tryptophan from histones is important in another connexion, for while histones can be readily distinguished from chromosomins by their general chemical and physical properties, they are also distinguished by the fact that chromosomins are rich in tryptophan.

As indicated by the name chosen for it, the view is held that chromosomin is the essential component of the chromosomes. Many facts in support of this view have been discussed in detail elsewhere; they can be summarized in the statement that chromosomin is the only known nuclear component possessing the requisite properties for fulfilling this function. The alternative, that chromosomes are composed of histone or protamin nucleate,

has been shown to be unsatisfactory from many points of view. If further proof were required that these basic proteins form no essential part of the chromosomes, it is provided by the results of our investigation, mentioned above, on the cell nuclei of the salmon. In this species, the basic protein of the sperm head is entirely different in composition from that in the somatic nuclei; in the former it is a protamin, in the latter a histone. Unless the principles of cytology and genetics are to be abandoned, this result irrevocably disposes of the older view concerning the functions of protamins and histones in the nucleus.

Whether or no chromosomin is the sole constituent of the chromosomes is a question upon which our experiments obviously throw no light. They do not, for example, exclude the participation of nucleic acid in their formation. Moreover, if nucleic acid does enter into their structure, our experiments give no indication of the proportion of the material involved. Nevertheless, there is substantial evidence that the nucleic acid is by no means wholly concentrated in the chromosomes. Material must be available to form the nuclear sap and the spindle to which it gives rise during mitosis, and, apart from nucleic acid there is, in many nuclei, insufficient material present for this purpose. It is therefore suggested that nucleic acid forms the matrix of the nucleus, in which structures such as the chromosomes are embedded. In this case it is clear that any chromosomin exposed at the extensive surface of the chromosomes would enter into combination with the nucleic acid of the matrix. It is a moot point whether or no this nucleic acid would have to be regarded as a component of the chromosomes.

The basic proteins, in combination with nucleic acid, no doubt contribute to the formation of the nuclear sap, the properties of which would vary with the proportion of protein present. Limitations of space preclude a discussion of this important point, but data will be presented illustrating the variation in histone content of various types of nuclei. This data is in accordance with the hypothesis, advanced elsewhere, that the histone content of nuclei is an important factor in the control of cell proliferation.

Cytoplasmic Ribonucleoproteins. By J. N. Davidson

It is now recognized that the animal and plant cell nucleus contains deoxyribonucleic acid while the cytoplasm contains a pentose polynucleotide which is usually referred to as ribonucleic acid although the pentose has been proved to be ribose only in the cases of the polynucleotides from yeast and from liver. In many tissues, e.g. pancreas, liver, and intestine, the concentration of cytoplasmic ribonucleic acid is considerable and exceeds that of nuclear deoxyribonucleic acid.* The presence of ribonucleic acid in the cell cytoplasm can be clearly shown by a histochemical test, first described by Brachet,† which makes use of the fact that ribonucleic acid, but not deoxyribonucleic acid, is attacked by the enzyme ribonuclease. In some tissues, e.g. liver, the cytoplasmic ribonucleic acid stains deeply with such basic stains as pyronine or toluidine blue. Treatment with crystalline ribonuclease removes the ribonucleic acid and the cytoplasm is no longer basophilic although nuclear staining is unaffected.*

Ribonucleic acid occurs in the cytoplasm in the form of phospholipin-ribonucleoprotein complexes which exist as particles or granules of two main sizes—large particles 0·5–2·0 μ. diam. and small particles 50–200 mμ. diam.‡ The large particles form the mitochondria and the secretory or zymogen granules of the liver and pancreas, while the small particles or 'microsomes' form the chromophilic ground substance of the cell. The presence of both types of particle has been conveniently demonstrated by submitting small fragments of fresh tissue to high speed centrifugation.†‡ Liver cells so treated, when fixed and stained, show four separate layers. The lowest layer, at the centrifugal pole, represents glycogen, the second layer contains the nucleus and the large particles, the third layer consists of the small particles or chromophilic ground substance and the top layer is

composed of very lightly staining true hyaloplasm. By a process of differential centrifugation the particles can be isolated from tissue extracts and submitted to chemical analysis. Both types of particle contain ribonucleic acid, phospholipin (including acetal phospholipin), and protein rich in —SH groupings. The particles contain nearly all the phospholipin of the cell cytoplasm,§ and in the adult cell, nearly all the ribonucleic acid. In the chick embryo, in yeast, in amphibian eggs in course of development, and in young oocytes some 60–80 % of the total cytoplasmic ribonucleic acid has been reported to be present in 'free' form which is non-particulate in nature and not sedimentable in the ultracentrifuge.†

Some of the cytoplasmic particles are endowed with specific biological activity. For example phospholipin-ribonucleoprotein complexes isolated from lung tissue have thromboplastic activity. In other tissues, e.g. liver, the particles may form part of a store of labile protein. In the liver of the rat, fasting is accompanied by a rise in the total nucleic acid concentration, the proportion of ribonucleic acid falling while that of deoxyribonucleic acid rises.* Calculation of the nucleic acid content of the whole organ reveals that the ribonucleic acid content shows a marked fall while the deoxyribonucleic acid remains unaltered.

Effect of fasting on the liver of the rat

	Fed	Fasted 24 hr.
Weight of liver (g.)	10·6	7·9
Weight of liver as % body weight	2·9	2·5
Total nucleic acid P concentration: mg./100 g. fresh tissue	134	152
mg./100 g. dry tissue	449	502
Ribonucleic acid P per liver (mg.)	10·7	7·9
Deoxyribonucleic acid P per liver (mg.)	2·5	2·4

Moreover, fasting is followed by the loss of phospholipin and of protein from the liver, by a reduction in cytoplasmic volume, and by a disappearance of granular material from the cytoplasm. These facts would be consistent with the view that a reserve of protein is stored in the liver as phospholipin-ribonucleoprotein complexes in the cytoplasm.††

Another possible function of cytoplasmic ribonucleoproteins is to act as a source of smaller nucleotide molecules. Fragments of some of the large phospholipin-ribonucleoprotein complexes of lung tissue may retain part of the original thromboplastic activity. It has also been suggested that the ability of ribonucleoproteins to cause neural induction when implanted in the amphibian embryo may be due to the gradual liberation of active fragments.† More drastic breakdown might yield substances with coenzyme activity such as guanine which functions as a coenzyme in transphosphorylation processes.¶

The phospholipin-ribonucleoprotein particles of cell cytoplasm are endowed with enzyme activity. Succinic dehydrogenase and cytochrome oxidase are among the enzymes found in the particles but not in the interparticulate fluid.†§ Since the particles also contain proteases, e.g. dipeptidase, it has been pointed out that they constitute excellent organs for protein synthesis,† and Claude has suggested that they may be endowed with the property of self-duplication.‡ Indeed a combination of protein with polynucleotide appears to be a distinguishing feature of self-reproducing systems, cf. the genes. It appears probable, therefore, that the phospholipin-ribonucleoprotein macromolecules constitute some of the fundamental units out of which living matter is built. They also provide a link with the viruses, some of which contain ribonucleic acid either in the form of ribonucleoprotein alone, e.g. tobacco mosaic virus, or in the form of phospholipin-ribonucleoprotein complexes, e.g. Rous sarcoma virus, equine encephalomyelitis virus.

The view that the cytoplasmic particles are organs for protein synthesis is in accordance with the observations that a high ribonucleic acid content is characteristic of tissues in which rapid protein synthesis is taking place either for purposes of cell multiplication or

for secretion.*†** Thus a high ribonucleic acid concentration is found in the pancreas, liver and gut, in the tissues of the embryo and in certain tumours. According to the Caspersson school, protein synthesis is governed by the heterochromatin of the nucleus which controls the synthesis of histones. These histones accumulate in the nucleolus and diffuse out towards the cytoplasm. As they reach the nuclear membrane, they induce the formation of cytoplasmic ribonucleic acid which in turn controls the synthesis of cytoplasmic protein.**

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The Inorganic Constituents of the Cell. By E. J. Conway

Regarding the cell as a centre of energy change, Fe would no doubt be primarily considered among its inorganic constituents. On the other hand, if the cell is thought of in terms of form and structure, Fe would lose its predominant position. It is proposed to deal here with such constituents in a purely quantitative order, avoiding thus a prejudgement of their relative significance, and only some comparisons of concentrations and modes of accumulation can be touched upon.

The inorganic composition of cells. For cells in general K is the outstanding cation, just as Na is that of the external environment of the cell. It will be convenient here to refer to a particular example of cellular tissue such as skeletal muscle quantitatively the most important soft tissue in higher animals. In a kg. of rat muscle fibre water there are 153, 17, 6·5 and 1·8 mM. of K, Na, Mg and Ca; the concentration ratios with respect to the external plasma water being 25, 10, 0·6 and 0·12 (Conway & Hingerty, 1945). The H ion ratio across the membrane appears to equal that of K (Conway & Fearon, 1944).

With these we may compare figures for Zn, Fe and Cu, from the data of Eggleton (1940), Sherman (1923) and Tompsett (1940). Per kg. of fresh muscle there are approximately 0·8, 0·5 and 0·02 mM. respectively, the plasma figures being 0·2, 0·03 and 0·06 (the Zn and Cu figures being given for blood by Eggleton, 1940). Thus Fe would appear relatively as concentrated as K though we may be sure that almost all the Fe is in organic combination. Traces of many other elements are present in cells as shown in such studies as those of Vinogradov (1935) and of Fearon & Webb (1937) and have occasionally special importance.

As to the inorganic anions, Cl, HCO_3 and presumably phosphate are in muscle of the expected order for a Donnan relation with respect to K^+ and H^+ (Conway & Fearon, 1944; also Wilde, 1945, when allowance is made for the non-penetration of the red corpuscles by inulin.) It may be noted that the total acid-soluble P in rat skeletal muscle has a mean value of 93 mM./l. of fibre water.

Such is the general quantitative picture for muscle, and largely applicable to other cells. Only some outstanding points can be briefly considered.

An answer to the question of cellular predominance of K. K as an ion in solution is smaller than Na, because of the water of hydration, and the size of the hydrated ions go in the

order K, Na, Li, Mg and Ca. It will also appear that if K or Na enter a cell in relatively high concentration it must do so with anions. (It is conceivable that such a conclusion could be avoided by certain assumptions which need not be considered here.) With a membrane permeable then to cations and anions we may write (Conway, 1941)

$$\eta = c - \sqrt{(\epsilon^2 + 4\Sigma bd)},$$

where η is the sum of the non-diffusible molecules which the cell must retain and ϵ the balance of negative charges thereon (expressed as m.equivs. alkali that would satisfy it) b and d being the diffusible cation and anion concentration outside. If, at the same time, the external environment be similar to frog plasma (c being the total concentration) and the membrane permeability be such that Na and K are both allowed through as well as small anions (Cl, HCO_3, etc.) then it may be calculated that even when ϵ is zero there are only 11 mM. available for the non-diffusible constituents. If the permeability be so gauged that Na is excluded but K allowed through then 197 mM. become available. Clearly, the adjustment of the permeability to exclude Na and allow K to pass would be of great advantage to the cell. By means of this exclusion, when cellular growth occurs it may be shown that K will be automatically accumulated with phosphate esters and protein. If we suppose that the cell in an early marine environment developed its internal composition and an ionic permeability as above, such a condition may be taken as a standard pattern followed in greater or lesser similarity in subsequent multicellular developments. The permeability whereby Na is excluded and K admitted, has been termed the 'standard permeability for ions' by the author (Conway, 1945). Exceptions to it can no doubt be pointed out, but if we consider the permeability to apply only to the formative period of the cell these may be mostly only apparent.

The accumulation of Mg, Ca, *etc.* An ionic permeability excluding Na will naturally exclude Mg, Ca and other ion; but unlike Na these can form unionized complexes which may enter the cell, just as in general lipoid soluble substances can enter. Such complexes —as with Ca—may even be included in the membrane. The considerable difference between Mg and Ca and other metals may be interpreted as arising from solubility relations of the complexes formed, and because of these complexes the chemical potentials of such salts as $MgCl_2$, $CaCl_2$, etc. are not the same inside as outside. Also, marked localizations of these substances may occur.

Localization. Concerning localization, perhaps the most interesting development is that of micro-incineration, a recent account of which has been given by Horning (1942), and which of late years has been developed mostly through Policard, Scott and co-workers. So far Na and K do not appear readily amenable to such study and are lost in the incineration. In the published plates of Scott (1933) Mg may be assumed to account for most of the ash. More recently Scott & Packer (1939) have combined the use of the electron microscope with the micro-incineration technique.

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The **246th Meeting** *of the Biochemical Society was held in the Bearsted Theatre, London Hospital, Whitechapel Road, London, E.* 1, *on Saturday,* 8 *December* 1945, *at* 2.15 *p.m., when the following communications were given:*

COMMUNICATIONS

The Detection of 3-Hydroxysulphanilamide in the Urine of Patients Treated with Sulphanilamide. By R. T. WILLIAMS and I. ANSELL

3-Hydroxysulphanilamide has been isolated (Williams, 1945) in a pure crystalline state from the urine of rabbits dosed with sulphanilamide. It has now been detected in the urine of hospital patients treated with sulphanilamide. Urine was collected from three patients who had received 26, 24·5 and 44 g. respectively of sulphanilamide. The first two were being treated for urinary infections and the third for a haemolytic streptococcal infection. They also received potassium citrate, potassium bicarbonate and sodium acetate; the third patient had, in addition, sodium salicylate.

The urine in each case was taken to a small volume *in vacuo*, then hydrolyzed by HCl, neutralized and extracted with ether in a continuous extractor. The extract was evaporated and as much sulphanilamide as possible removed by crystallization. 3-Hydroxysulphanilamide was concentrated and separated from sulphanilamide and from salicylic acid by chromatography on alumina. The hydroxysulphanilamide was located in a yellow band, which was extracted with hot water. The aqueous extract was shaken with peroxide-free ether in the presence of dilute $FeCl_3$. The presence of 3-hydroxysulphanilamide was indicated by an intense ruby red colour in the ether layer whose absorption spectrum showed a band with a maximum at 530 mμ. All three urines gave positive results.

N-Acetylhydroxylaminobenzenesulphonamide (red), 2-hydroxysulphanilamide (weak purple) and salicylic acid (intense purple) give colours with $FeCl_3$ in aqueous solution but the pigments are insoluble in ether. *p*-Aminophenol gives an intense purple $FeCl_3$ reaction but the ether extract has only a weak red colour with a band at 510 mμ.

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Some New Evidence on the Dynamics of Red Blood Cell Destruction. By E. M. JOPE

Methaemoglobin (MHb) disappears rapidly from circulating blood after the causative agent (T.N.T., sulphonamides, etc.) has been removed, being no longer detectable after about 2–3 days; in drawn blood at room temperature it may disappear in as little as 24 hr. This reversion to functional haemoglobin is arrested by iodoacetate and fluoride and is probably coupled to the similarly inhibited reducing systems of the intact red cells, such as glycolysis (Drabkin, 1945). Continued raised levels of MHb in toxic cyanoses must be due to a preponderance of toxic oxidative processes over the intracellular reducing processes. By contrast sulphaemoglobin (SHb) persists for many weeks. A probable explanation of this is that the red cell contains no mechanism capable of changing SHb back into functional haemoglobin, and that the body has no means of removing SHb other than by destroying the red cells that contain it. The disappearance of SHb was accordingly studied in seven cyanosed T.N.T. workers* over 4 months in an attempt to follow the dynamics of this red cell destruction.

* By courtesy of the Chief Medical Officer, Ministry of Supply, and Drs H. K. Tucker and Helen Ramsay.

Plots of the fraction of the initial SHb level against time were well fitted by a straight line cutting zero SHb at 116 days, which period, upon certain assumptions, may be considered as the life span of the red cells containing SHb. This linear relation indicates that these red cells have a definite life span and are not subjected to indiscriminate destruction. These conclusions agree closely with those of Callender, Powell & Witts (1945) using the Ashby differential agglutination technique, which involves assumptions very different from those made here, and has received some acceptance as giving normal red cell life span (*Lancet*, 1945). These two approaches are complementary. Rittenberg and Shemin (1945) have also recently confirmed, by means of the N^{15} isotope, that haemin, and therefore probably the red cell, has a life of at least 100 days.

This SHb method might be applied to the study of red cell destruction in animals, but complicating factors are such that a preliminary study of the formation of SHb *in vivo* is desirable. The use of the most precise spectrophotometric methods of estimation is strongly urged, in order to eliminate the possible interference of other pigments, and in such estimations it is essential that great care be taken to remove all turbidity from the blood dilutions used in the spectrophotometer.

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The Metabolism of Chrysene in the Rat. By I. Berenblum & R. Schoental

The study of the metabolism of polycyclic aromatic hydrocarbons is of interest, especially in connexion with the possibility that the metabolites may be implicated in the inhibition of the growth of tumours observed under the influence of some compounds of this class (Haddow, 1935, 1938; Haddow & Robinson, 1937, 1939; Badger, Elson, Haddow, Hewett & Robinson, 1942).

Previous studies (Boyland & Levi, 1936, 1941; Dobriner *et al.* 1942; Berenblum & Schoental, 1943; Berenblum *et al.* 1943) of the metabolism of some carcinogenic and related polycyclic hydrocarbons revealed that they undergo oxidation to phenolic derivatives in the animal body, and that the positions metabolically attacked differ from those which are known to be the most reactive in substitution reactions *in vitro* (e.g. the *meso*-positions in the group of anthracenoid substances).

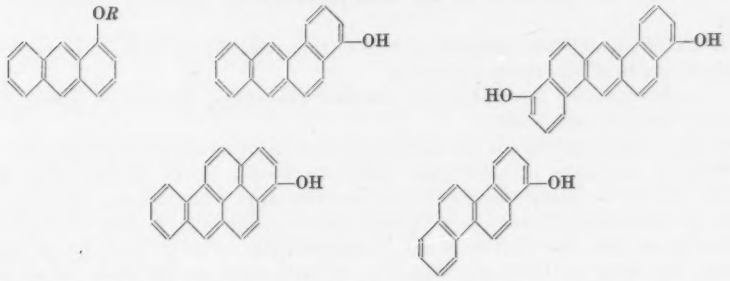
The study of the non-carcinogenic chrysene, which does not belong to the anthracenoid type and has a symmetrical structure, seemed of interest. Its position 2- is known to be the most reactive in mono-substitutions, while di-substitutions lead mostly to 2:8-derivatives (Neuman & Cathcart, 1940).

In order to facilitate the identification of the chrysene metabolite, all the six possible isomeric mono-chrysenols and some of their derivatives have been synthesized (Cook & Schoental, 1945).

The actual procedure of the isolation of the metabolite was essentially the same as in our previous metabolic studies, with appropriate modifications. Rats were injected intraperitoneally with chrysene in arachis oil, and the collected faeces were dried, ground and extracted with benzene in the cold. The extract was chromatographed on alumina, the phenolic zone cut, eluted and methylated. The methyl ether of the metabolite was purified by repeated chromatography and crystallized from petroleum ether. It formed white shiny leaflets and exhibited in solutions the fluorescence spectrum characteristic

of the synthetic 3-methoxychrysene. Confirmation of its being 3-MeO-chrysene was obtained from mixed melting-point with the synthetic 3-isomer, the formation of a crimson red complex with 2:7-dinitro-anthraquinone [m.p. 283° C., not depressed when mixed with the complex of the authentic 3-MeO-chrysene], as well as from a study of ultra-violet absorption spectra, carried out by E. R. Holiday and M. Jope.

Rat's metabolites of anthracene, 1:2-benzanthracene, 1:2:5:6-dibenzanthracene, 3:4-benzpyrene and chrysene.



In conclusion it can be said that also in the case of chrysene the metabolic oxidation does not take place in the most reactive position 2-, but in the position 3-, an α-position to the naphthalene nucleus, and leads to mono-substitution only.

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The Study of the Fine Structure of Absorption Spectra. By E. R. Holiday

The use of absorption spectrophotometry as an analytical method is limited almost entirely to the case in which the absorption spectrum is selective in type. Theoretically it is highly improbable that two compounds show identical absorption spectra. Where a selective absorption band is of the broad diffuse type the position of the maximum is only located with considerable error, and the specificity of the absorption band for a particular compound is low. The specificity is considerably increased by measuring, for a given concentration of the compound in solution, the distribution of absorption intensity throughout the band and then plotting this as extinction coefficient against wave-length or frequency—the so-called absorption curve.

Where the selective absorption is of the multi-banded type, the maximum of the bands, which in these cases are usually narrow, can be located with considerable accuracy and the specificity is high. In extreme cases in which ten to fifty bands are identifiable, little increase in specificity is gained by measuring the intensity of the bands.

In material of biological origin this latter property may be of use, since such material, during the course of purification, is usually contaminated with absorbing impurities rendering the first method of small value.

A method has been devised (Holiday, 1937) for detecting the position of so-called fine structure in absorption spectra. It is claimed for the method that:

(1) It is more sensitive in the detection of fine structure than any method depending on measuring optical density relative to wave-length.

(2) It reveals fine structure in the absorption spectra hitherto considered to be uniform.

(3) It makes possible the identification of certain compounds in the presence of considerable amounts of absorbing impurities.

(4) It makes possible the distinction between absorption spectra of closely related compounds hitherto not possible.

(5) It gives new material for the theoretical treatment of absorption spectra.

(6) Since it interposes no additional quartz or fluorite in the optical train to that present in the spectrograph, the method is therefore applicable to measurements to the limits of the wave-length range of a vacuum spectrograph with fluorite optical train.

Its limitations are first that it is only semi-quantitative in its capacity to estimate absorption intensity and secondly that it is of little value in the study of absorption spectra in which no fine structure is detectable. Its chief application is to the study of unsaturated cyclic compounds.

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The Polarographic Estimation of Steroid Hormones. Part 1. The Estimation of 17 Ketosteroids in Urinary Extracts. By JEAN BARNETT and C. J. O. R. MORRIS

A technique has been developed for the estimation of 17-ketosteroids in urinary extracts by polarographic analysis, based on the discovery of Wolfe, Hershberg & Fieser (1940) that the water-soluble condensation product between 17 ketosteroids and the Girard reagent T shows a characteristic polarographic wave with a half-wave potential at $-1{\cdot}36$ V., the wave span of which is, within certain limits, proportional to the concentration of the steroid ketone, and which is sufficiently sensitive to enable amounts of the order of 10 μg. to be measured accurately.

The polarographic waves of urine extracts of ketosteroids deviate from the standard curve with isodehydroandrosterone in two main respects. In the first place, the final discharge tends to occur at a lower voltage, so that the point of inflection of the wave is less clear cut. To overcome this difficulty, an improvement first suggested by Werthessen & Baker (1945) has been introduced; this involves a decreased rate of increase of applied voltage, with a resulting 'spread' of the wave. The second difficulty encountered has been that extracts rarely give a good base-line. There is evidence that ketonic material, possibly 3- and 20-ketosteroids, is the cause of the interference. This has been reduced to a considerable extent by preliminary oxidation of the extract with potassium permanganate which does not affect isodehydroandrosterone. 17-Ketosteroid waves of urine extracts will be exhibited showing the improvements obtained by these methods.

A preliminary estimation of 17-ketosteroids in the urine of eleven normal young males has been carried out. Results of these will be given.

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The Polarographic Estimation of Steroid Hormones. Part 2. Polarography of Related Steroid Hydrazones. By JEAN BARNETT and C. J. O. R. MORRIS.

In attempting to improve the technique described in Part I, a number of reagents were made, one of which it was hoped, might replace the Girard T reagent in forming water-soluble compounds and which might show more favourable properties in the type of wave-form exhibited on the polarograph. Accordingly, ketosteroid hydrazones of the following substances were studied: hydrazinoacetic and benzoic acids respectively; amino-, monomethylamino- and dimethylamino-acethydrazides; thiosemicarbazide; dimethylaminobenzene sulphonhydrazide; maleic acid monohydrazide and *p*-amino-benzoic acid hydrazide. With the exception of the first two mentioned substances, which apparently gave no wave, all the hydrazones gave waves similar in shape to the Girard T wave; the only hydrazones which showed half-wave potentials varying appreciably from the latter were the two sulphur-containing compounds. In no case was there any favourable improvement on the Girard T wave, although the polarograms obtained with aminoguanidine and with maleic acid monohydrazide were almost as good.

In view of these results, it is suggested that the structure of the reagent essential for this wave to be shown is

$$-X(=)-NH-NH_2,$$

which after condensation with a ketone group R'—C=O may yield, by enolization, the grouping

$$-X(R)=N-N=C-R',$$

where X is C or S and R may be —OH (as in the acid hydrazides), —NH_2 (as in aminoguanidine) or —SH (as in thiosemicarbazide). Hydrazinoacetic and hydrazinobenzoic acids which cannot conform to this structure apparently yield no waves.

A Case of Nutritional Oedema. By J. R. MARRACK

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